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Disentangling the effects of the Agulhas Current on marine viruses

By

Nyasha Mafumo

21620742

Supervisor: Prof. Thulani Makhalanyane

Co-supervisor: Dr. Oliver Mogase

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Declaration

I, Nyasha Mafumo, declare that the thesis/dissertation ‘Disentangling the effects of the Agulhas Current on marine viruses’, which I hereby submit for the degree Magister Scientiae Bioinformatics at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Chapter 1

Unveiling the ecological significance of viruses in the ocean: implications for microbial communities and biogeochemical processes

1. Summary

Traditionally, viruses were considered pathogens of marine organisms. These studies assumed that viruses occurred at low abundances in the oceans and had a limited influence on marine ecosystem function. However, over the past three decades, various studies have confirmed that marine viruses constitute the most abundant entities in the oceans, with current estimates suggesting approximately 10^{30} viruses globally. In addition to their ubiquitous nature, marine viruses are highly diverse. They infect a wide range of organisms including microorganisms and macrofauna. Consequently, viruses play a significant role in shaping the evolution, community structure and biodiversity of marine populations. Furthermore, there is clear evidence of the profound role played by marine viruses in the cycling of nutrients, organic matter, and energy. Here, we review current insights on the diversity and function of marine viruses. We focus on the impact of environmental variables on viral dynamics, including the viral shunt and the expression of genes that augment host metabolism (Auxiliary Metabolic Genes). We also explore the potential function of viruses in regions that are actively interacting with current climate change, such as the Agulhas Current and the Southern Ocean. Ultimately, this review provides valuable insights into the distribution and ecological contribution of marine viruses.

2. Introduction

Over the past three decades, there has been a notable resurgence in the study of viruses that infect microbes, termed bacteriophages (1, 2). These studies have focused on environmental viruses, including those found in the oceans (3-7). Increasing recognition of the importance of prokaryotic and eukaryotic plankton in primary production has likely driven renewed interest of bacteriophages (8-10). This is because viruses play a pivotal role in shaping the diversity, evolution, and metabolism of phytoplankton (11-14). Indeed, marine viruses account for 10% of phytoplankton mortality, with higher percentages during phytoplankton blooms, and more than 40% of total bacterial mortality (15-18). However, viral ecology is a relatively challenging field to study as viruses lack universally conserved marker genes (19).

Virus-induced mortality of microbes can affect ecosystem function (20, 21). Through the lysis of their hosts, viruses release large amounts of dissolved organic matter (DOM) back into the environment (21-23). This process is called the 'viral shunt' (12). The viral shunt diverts organic carbon along with other nutrients from the secondary consumers in the food web, to the microbial loop, where it is consumed by heterotrophic bacteria (15, 24). This leads to bacterial growth and increased respiration at the expense of organic carbon sinking (25, 26). On the other hand, viral infection has also been suggested to increase particle formation and biomass sinking (27). This potentially increases the sequestration of carbon into the deep sea, through a process known as the 'viral shuttle' (17, 28, 29). Despite the potential role of viruses in sequestering carbon into the deep sea, the quantitative impacts of viral infection on microbial community composition and carbon fate remains poorly understood (30).

Marine viruses have also been reported to influence the metabolic output of their hosts through the expression of auxiliary metabolic genes (AMGs) (31-37). AMGs are host-derived viral genes that modulate metabolic pathways in the infected organism to favour viral replication (32, 37, 38). AMGs comprise a diverse range of functions including photosynthesis, sulfur (S), carbon (C), nitrogen (N), phosphorous (P) and amino acid metabolism (39-43). Studying marine viruses and their associated AMGs is important because it provides insights on viral-host interactions (30) and ecosystem

dynamics (3, 44) . It also improves our understanding of viral influence on the biogeochemical cycling of elements (7).

In this review, we provide a synthesis of current literature on the distribution and function of marine viruses in the global oceans. Our emphasis is primarily on understanding the contribution of viruses on the biogeochemical cycling of elements (45). We also discuss environmental factors that potentially influence the distribution of viruses in two oceanic regions that are important in climate mitigation: the Southern Ocean and the Agulhas Current. The Agulhas Current is characterized by high wind speeds that contribute greatly to the moderation of rainfall and climate in Southern Africa (46-48). While the Southern Ocean takes up to 40% of anthropogenically produced carbon dioxide from the atmosphere (49, 50). The Agulhas Current and the Southern Ocean play a crucial role in the global climate system, and marine viruses are essential for shaping their ecosystems. Therefore, studying viral communities in these regions is of vast importance.

3. Viruses as the most diverse entities in the oceans

While several early studies provided evidence regarding the existence of viruses, our understanding regarding their numerical abundances emerged fairly recently (51, 52). Studies in the last 30 years, based on the use of transmission electron microscopy (TEM), revealed the high abundance of viruses (~10^7) (51-53). Indeed, recent estimates using epifluorescence microscopy (EFM), flow cytometry (FCM), and transmission electron microscopy (TEM) have shown that viral abundance ranges between 10^6 to 10^8, depending on the region of the ocean (54, 55). Estimates of viral abundance have contributed in understanding the spatiotemporal distribution of viruses, their interactions with hosts, and their role in ecosystem functioning (37, 54, 56-58). These studies have provided evidence that viruses play a significant role in the mortality of a diverse range of organisms, and contribute to the functioning of natural ecosystems.

In addition to the ubiquity, viruses are the most diverse entities in the oceans (59, 60). Previous work suggest that this high diversity may be driven by rapid replication and

high mutation rates (61-63). There is also evidence that this high diversity is due to the ability to acquire genetic material from their hosts, and other viruses in the environment (64, 65). Marine viruses come in a plethora of shapes, sizes, and molecular composition (30). The viruses can be helical, icosahedral, elongated, or lemon-like in shape (37, 66). The genetic material can either be single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) (67), single-stranded DNA (ssDNA) (68), or double-stranded DNA (dsDNA) (17, 69). Moreover, the viruses can be tailed or non-tailed, with the size measuring from 20 nm and 1.2 µm in diameter (37, 70).

The vast majority of marine viruses are thought to be bacteriophages (1, 2). dsDNA viruses are the most abundant type of viruses in public databases (71). dsDNA viruses infect a variety of marine organisms including prokaryotes, eukaryotic phytoplankton and zooplankton (72-74). Among dsDNA are giant viruses. Giant viruses encode thousands of proteins, including proteins that were thought to be limited to cellular organisms (75-77). Giant viruses infect a broad range of eukaryotic hosts, including animals and diverse unicellular eukaryotes (78, 79). On the other hand, ssDNA viruses have a smaller genome size, and infect both prokaryotes and eukaryotes (80). RNA viruses primarily infect eukaryotes including protists, fungi and invertebrates (81). These viruses are among the least studied due to the instability of RNA (82). However, there has been increasing evidence of high abundance and diversity of RNA viruses in global oceans (83-85). These findings show that marine viruses are very diverse and they infect a broad range of hosts across the tree of life.

4. Forms of viral replication

Marine viruses replicate in a diverse range of ways, with different viruses and hosts employing different strategies (7). However, the two major modes of viral replication are temperate and lytic bursting (86). In lytic infection, the virus injects its genetic material into the host cell, which redirects the host mechanism to replicate the viral genome (87). Eventually, these viral particles are released from the host cell, resulting in cell death. These particles are released into the surrounding water, where they can infect other hosts (3). Temperate viruses can replicate in two ways, either using the

lytic cycle or lysogenic cycle (88). In lysogenic replication, the virus can integrate its genetic material into the host genome or forms either circular or linear plasmid within the host cytoplasm (88, 89). The viral genome can remain dormant as a prophage in the host cell until an environmental or cellular trigger causes them to enter the lytic cycle (87). Previous work suggests that temperate viruses are widespread, with some studies reporting that more than 90% of known bacteriophages are temperate viruses (90). Indeed, J. R. Brum et al. (91) revealed that temperate viruses dominate the Southern Ocean.

It has long been recognised that viral infection transforms host metabolism (37, 38, 92). During lytic infection, the host metabolism is altered to promote viral reproduction (7, 87). This augmented reproduction is evident in the shift in mRNA synthesis from host metabolic processes to viral production (93-95). Moreover, lytic phages can contribute to the selection of bacterial communities by killing competitor bacterial strains and releasing their cellular contents back into the environment (96). On the other hand, lysogeny has complex and long-term impacts on the ecology and evolution of the host. The incorporation of the viral genome into the host genome can alter gene expression and function, which can change the host's metabolism and cellular processes (97, 98). These processes can be virulent factor production or immunity to infection by homologous viruses (92). Furthermore, temperate phages can facilitate the transfer of bacterial DNA, which can potentially confer new phenotypes, such as antibiotic resistance (99). Therefore, viral infection has a profound effects on host metabolism, regardless of the mode of infection.

5. Distribution and abundance of viruses in the oceans

Viral concentrations vary widely across different marine environments. In surface waters, estimates typically range from 10^6 to 10^9 mL^{-1} (17, 54, 55). In contrast, the mesopelagic and deep ocean regions have lower viral concentrations, with estimates ranging from 4 x 10^4 to 10^7 mL^{-1}, respectively (55, 100, 101). Marine sediments have even higher viral abundances than seawater, with estimates ranging from 10^7 and 10^{10} viruses per gram of dry weight (26, 102). Notably, coastal, low-salinity waters

also have higher viral abundances (103, 104), in comparison to open waters (105) and deep sea sediments (102).

Viral abundance has been shown to correlate with primary production (103, 106). Primary producers can influence viral dynamics and abundance in both direct and indirect ways (26). Directly, primary production can affect viral abundance by influencing the populations of potential host organisms. For example, phytoplankton blooms can lead to increases in viral abundance, as there are more host cells available for infection (18, 107, 108). Indirectly, primary producers can influence viral dynamics by providing organic resources to heterotrophic hosts (17, 109). For example, the release of dissolved organic matter (DOM) from phytoplankton can support the growth of heterotrophic bacteria (110), which can then serve as hosts for viruses.

Early studies predicted viruses to be 10-folds more abundant than bacteria (111, 112). However, there is accumulating evidence showing that viral-host levels vary and may not be accurately described by the 10:1 ratio (10 viruses for every 1 bacterium) (109, 113, 114). These studies indicate that the virus-to-bacteria ratio (VBR) exhibits variation across different environments, with estimates typically falling within the range of around 5 to 10 virus-like particles per bacterium (109, 113, 114). These differences suggest that the VBR is potentially affected by environmental variables. Indeed, a study by J. F. Finke et al. (115) showed that environmental variables, are equally or even more important than host abundance in influencing the quantities of viruses. Furthermore, the analysis of viral morphology, in the world's oceans, confirmed that a suit of environmental properties provided a better explanation regarding the differences observed in the distribution of viruses (60).

6. Physiochemical properties and geography influence viral abundance

Temperature and salinity

Temperature and salinity have been shown to affect the abundances and community composition of viruses (60, 116, 117). A rise in temperature, for example, may favour

the transition from lysogenic to lytic cycles, which influences viral distribution (118-120). Indeed, J. Shan et al. (119) revealed that at higher temperatures (37°C), some bacteriophages predominantly go through a lytic cycle, but at lower temperatures (25°C), the viruses remain temperate. Another example, reported from studies on microalgae, revealed that viral lysis occurred at temperatures between 20^{o} to 35^{o} (121, 122). Additionally, viral abundance has been shown to positively correlate with temperature in tropical estuaries (123).

High salinity levels trigger a shift in viruses from the lysogenic cycle to the lytic cycle, leading to an increase in viral abundance (124, 125). For instance, the marine phage ϕHSIC undergoes this transition when salinity levels exceed those found in its natural environment (124). Furthermore, several studies have reported that the abundance of bacteriophages positively correlated with salinity levels (125, 126). However, it has also been reported that bacteriophages can enter the lysogenic cycle under high-salinity conditions (127, 128). These contrasting effects of salinity on the phage life cycle can be attributed to the substantial variability in how salt concentration influences the attachment of phages to their host organisms (129). This variability depends on the specific characteristics of each individual phage (129). These findings highlight the significant role played by temperature and salinity in shaping the distribution of marine viruses.

Nutrient availability

Viruses primarily consists of a genome and a capsid (130, 131) and their stoichiometry differs from host cells (5). The high composition of protein and nucleic acids leads to an enrichment in of nitrogen and phosphorus in the virus compared to the host (132, 133). For example, the elemental stoichiometry of *Paramecium bursaria Chlorella* virus 1 (PBCV1) is estimated to be 17:5:1 (C:N:P) (134), compared to the baseline Redfield ratio of 106:16:1 for marine plankton (135). This indicates that PBCV1 is enriched in phosphorus and nitrogen relative to its host, the alga *Chlorella* NC64A (134). Furthermore, L. F. Jover et al. (5) demonstrated that up to 87% of cellular phosphate is incorporated into viral particles during infection. This highlight the importance of nitrogen and phosphorus in viral replication. Thus, viruses need to concentrate these essential nutrients in order to replicate. Indeed, previous work has

reported a positive correlation between the concentration of phosphorus and nitrogen in the environment with viral abundance (136-140).

Viruses obtain the nutrients they need for replication from both the host cell and the extracellular environment (133, 141). This has been well-established for phages infecting Escherichia coli, such as T2, T4, and T6 (142, 143). These phages derive phosphate and nitrogen from both the host biomass and the surrounding environment (142, 143). These early studies suggest that approximately 70% of phosphorus in progeny phage DNA, and 91% of nitrogen in the phage protein, is derived from the medium (142). This indicates that some viruses still need extracellular nutrients even when their hosts are not starving. Indeed, J. R. Waldbauer et al. (141) demonstrated that cyanobacterial phages obtained nearly half of their nitrogen from the environment, after infection. In contrast, the host proteins had almost no incorporation of this environmental nitrogen (141). This finding suggests that marine phages may have adapted to oligotrophic environments by evolving mechanisms to acquire nutrients from the environment.

7. Methods used to identify, characterise and quantify marine viruses

Microorganisms were studied using culture-based methods in the past, but these methods underestimated the number of microbes present in the environment by 100–1000-fold (144). This limitation was overcome by the development of 16S rRNA gene sequencing, which allows for the identification of prokaryotes by targeting a conserved region of their genome (145). Although 16S rRNA sequencing transformed microbial diversity studies and ecology (146, 147), viruses lack universally conserved marker genes (19). As a result, viral diversity and distribution have been studied using marker genes that are specific to certain taxonomic groups. For example, cyanophages have been intensively studied using conserved sequences of the capsid assembly protein gene (*g20*) (148-150). Another example of this is the RNA-dependent RNA polymerase (*RdRp*) gene that is used to identify picorna-like viruses (151). This

method however limits studies of the diversity of entire viral communities to genome fingerprinting (152).

Metagenomics-based studies were introduced to viral ecology in the early 2000s to address the challenges of culturing viruses and the lack of a universal marker gene for viruses (58). Metagenomics involves collecting and sequencing all the DNA in a particular environment, including viral DNA (153). Advances in sequencing technology and the reduction in cost, has made this approach relatively affordable. In metagenomic viral discovery, two main approaches are used to sequence viral genomes: bulk metagenomic sequencing and viral metagenomic sequencing (154). Bulk metagenomic sequencing is used to sequence the DNA of all the organisms in a sample, including bacteria, archaea, fungi, and viruses (155, 156). However, a central issue that has limited the application of bulk metagenomic sequencing to viral studies is the low quantity of viral nucleic acid compared to bacteria DNA (157, 158). Viral metagenomics involves using size filtration to select for virus-like particles, followed by viral DNA extraction and sequencing of the fragmented nucleic acid (159, 160). Short-read, high-throughput sequencing technologies such as Illumina sequencing are been proven effective for sequencing viral metagenomes (57, 161, 162). However, due to the relatively small size of viral genomes, long-read sequencing technologies, such as PacBio or Oxford Nanopore Technology, are emerging as good alternatives (163, 164).

To obtain sufficient viral nucleic acid for metagenomic sequencing and to minimize contamination by bacteria and extracellular nucleic acids, enrichment steps are typically performed after DNA extraction (158, 165, 166). These steps typically involve concentrating viruses from environmental samples, followed by purifying the concentrated viruses to reduce contamination (166-168). Tangential flow filtration (TFF) is the most commonly used method for concentrating viruses from seawater (169). TFF is efficient for concentrating viruses from large sample volumes, but it has several disadvantages. First, the filters are expensive (170). Second, the method is not well-suited for field use as the equipment is bulky and requires a power supply. Third, the recovery of viruses can be highly variable, ranging from 2–98% of viruses (171-173).

Iron chloride flocculation on the other hand is a relatively inexpensive, simple and effective method for concentrating viruses from seawater, with recovery rates typically exceeding 90% (167, 173). When iron (Fe) is added to seawater at a neutral pH, it forms iron oxyhydroxide particles which bind negatively charged viruses to form aggregates (168). These aggregates can then be filtered out of the water and the viruses eluted using a suitable buffer (168, 173). Dataset produced after enrichment of viruses offer several key benefits, including the ability to achieve robust de novo assembly for both abundant and rare viruses (161). It also increases confidence that the assembled sequences are actually viral genome (161).

Viral metagenomics has several advantages over traditional methods, such as culturing, electron microscopy, and molecular biology. Metagenomics does not require culturing hosts and isolating viruses, which can be difficult and time-consuming. However, metagenomics has revealed that the vast majority of viruses are uncultured and incredibly diverse (174). This has left the field with the challenge of understanding the factors that contribute to this great diversity (175). For example, in some marine studies, up to 93% of virome do not match any reference sequences in publicly available datasets (176). This limits the ability of metagenomics to provide insights into these viral communities.

To combat this issue of unknown sequences, protein clustering methods have been implimented (177). Protein clusters are defined based on the similarity of open reading frames (ORFs), with reference proteins (178). No prior knowledge of the taxonomy or function of the unknown sequences is required to create these clusters (157, 178). These methods have been identified as valuable tool for assessing viral diversity, community structure, evolution, and function at a global scale (156, 179, 180).

8. Marine phytoplankton: vital CO_2 consumers that are susceptible to viral infection

Biological and microbial carbon pumps

Marine phytoplankton account for half of all photosynthesis on earth, and they reside in well-lit regions of the ocean (8, 181, 182). The majority of phytoplankton are

microscopic unicellular prokaryotic and eukaryotic organisms with sizes ranging from 0.4 µm to 200 µm (182). Among the known marine phytoplankton taxa, diatoms, dinoflagellates, and haptophytes dominate phytoplankton communities (183-185). These taxa are responsible for seasonal blooms in temperate and polar waters (182). However, in oligotrophic regions of the ocean primary production is dominated by picophytoplankton (186, 187), particularly the cyanobacteria *Synechococcus* and *Prochlorococcus* (188, 189). The small size of these cyanobacteria gives them a competitive advantage for scarce nutrients in warm and nutrient poor water (190, 191).

Phytoplankton consumption of atmospheric carbon dioxide (CO_2) plays a vital role in regulating global climate (192, 193). The ocean sequesters one forth to one third of CO_2 produced from anthropogenic activities (194-196). The sinking of CO_2 from the atmosphere into the oceans is mainly due to the activity of the solubility and carbon pumps (197, 198). The solubility pump is a physio-chemical process that transports dissolved inorganic carbon in the deep oceans (197, 199). It is dependent on the solubility of CO_2 in surface seawater (200). The cold surface waters sink to the deep ocean, carrying their dissolved CO_2 with them (197, 200).

The biological pump is a biogeochemical process driven by marine biota that sequesters inorganic and organic carbon fixed by phytoplankton from surface waters to the deep ocean (197, 198, 201, 202). Phytoplankton photosynthesis removes CO_2 from the upper ocean, creating a difference in CO_2 concentration between the atmosphere and the ocean (203). This difference in concentration, or gradient, drives the absorption of CO_2 from the atmosphere into the ocean (203). Furthermore, photosynthesis by phytoplankton produces particulate organic carbon (POC) in the epipelagic zone (204, 205). Microbes, zooplankton, and their consumers process POC into fecal pellets and organic aggregates that sink into the deep ocean (206). Therefore, it is important to understand how viral infection affects phytoplankton community structure and function. This is because viral lysis and metabolic reprograming have implications for carbon sequestration and therefore climate change (3, 26).

9. Marine viruses as the primary drivers of biogeochemical cycling

Carbon, hydrogen (H), nitrogen, oxygen (O_2), sulfur, and phosphorus are essential elements for life and are the primary building blocks of biological macromolecules (207, 208). These elements circulate through ecosystems through biogeochemical cycles (209). Biogeochemical cycles are processes that allow chemical elements to be recycled between living and non-living components of the environment (209, 210). Global biogeochemical cycling is regulated by the key collective metabolic processes of microorganisms, such as sulfur metabolism, methane metabolism, carbon fixation, and nitrogen fixation (203, 208). For example it is well known that nitrogen is essential for all forms of life because it is required for nucleic acid synthesis e.g. DNA, RNA and amino acids (211). However the atmospheric nitrogen (N_2) is relatively unstable and cannot be used by biological organisms (212). Hence prokaryotic microorganism with the enzyme nitrogenase have to 'fix' this nitrogen before it can be used as ammonia (212). Therefore, microorganisms have recognised as drivers of biogeochemical cycles (208, 213).

Viruses influence the cycling of elements by altering microbial evolution through horizontal gene transfer and the viral shunt (33, 36, 214, 215). Viral mediated altering of evolution has been demonstrated in marine cyanobacteria. Cyanobacteria play an important role in the global carbon cycle by fixing CO_2 to organic substances through photosynthesis (216, 217). D. Lindell et al. (218) showed that viruses can facilitate gene transfer between different strains of the cyanobacterium *Prochlorococc*us. This gene transfer can lead to the evolution of novel strains of *Prochlorococc*us with new traits, such as the ability to photosynthesize more efficiently (218). Viruses can also alter the community structure of marine cyanobacteria. M. B. Sullivan et al. (36) investigated the effects of viral infection on the community structure of marine cyanobacteria. Indeed, their results depicted that viruses have the capability to eliminate dominant cyanobacterial species (36). This creates opportunities for other species to thrive and assume their ecological role (36). This alteration in the community structure has profound effects on the transfer of carbon across the marine environment.

Viruses possess and express Auxiliary Metabolic Genes during infection

The influence of viruses on biogeochemical cycling begins at the moment of infection as the virus remodels host metabolic processes and expresses AMGs (13, 33, 36, 219). AMGs are genes that are found in the viral genome but were are acquired from cellular hosts (37). These genes were previously thought to be only found in cellular genomes (7, 37, 39). Bacteriophages that infect marine cyanobacteria *Prochlorococcus* and *Synechococcus,* contain and express host like photosynthesis genes during infection (35, 36, 220). Viruses express these genes during infection in such a way that augments key steps in the host metabolism and ensures their own success (36). For example, it has been hypothesised that aquatic cyanophage replication is limited by energy availability (221). Thus, carrying and expressing AMGs that are relevant or associated in energy metabolism gives a fitness advantage through photophosphorylation (221).

Despite their name, AMGs do not only encode proteins with metabolic functions. AMGs can be genes that regulate other host functions such as motility and transport (92, 222). As a result, AMGs can be classified into two categories, Class I and Class II AMGs (39). Class I encode proteins that have metabolic functions such as photosynthesis, sulphur metabolism, carbon metabolism and amino acid metabolism (41, 223, 224). Class II genes are not represented in KEGG metabolic pathways because they have only a peripheral role in metabolism (39). These includes genes involved in membrane transport, vitamin and cofactor synthesis, iron-cluster assembly, and stress response (225-227). However, the study of AMGs is a relatively new field (37) and we are only beginning to understand their contribution in biogeochemical cycling.

Auxiliary Metabolic Genes modulate nutrient acquisition in nutrient limited environments

Viruses exploit extracellular resources to alleviate resource limitation during infection (225). This is clearly reflected in AMGs involved in nutrient acquisition. Cyanophages, for example, have phosphorus acquisition genes that are upregulated during infection when the hosts are phosphorus starved (228). Moreover, AMGs for phosphorus acquisition are more prevalent in viral genomes from phosphorus limited regions of

the ocean, where the hosts are likely to have limited intracellular phosphate (229, 230). In phosphorus-limited environments, viruses use AMGs to alter the host's metabolic pathway in such a way that increases phosphorus levels within the host and facilitates viral survival (225).

Moreover, a nitrogen transporter gene has also been identified in OtV6, a virus that infects the algae *Ostreococcus Tauri* (231). During infection, the viral protein can transport ammonium, methylammonium, and as well as organic nitrogen substrates into the host (231). AMG involved in other steps of the nitrogen cycle like nitrification, denitrification and anammox have been reported before as described in the review by (232). These studies clearly show that viruses play an active role in the biogeochemical cycling of elements by modulating how their hosts acquire nutrients.

Contribution of viral lysis to biogeochemical cycling

The viral shunt contributes significantly to the cycling of elements in the marine environment (12, 233). Viral lysis of microbial cells releases cellular contents, such as carbon, nitrogen, and phosphorus, back into the environment (15, 24). Thus, the dissolved organic matter (DOM) that would otherwise be converted into a form that can be consumed by higher trophic levels by the microbial loop is redirected back into the environment (20, 23, 234).

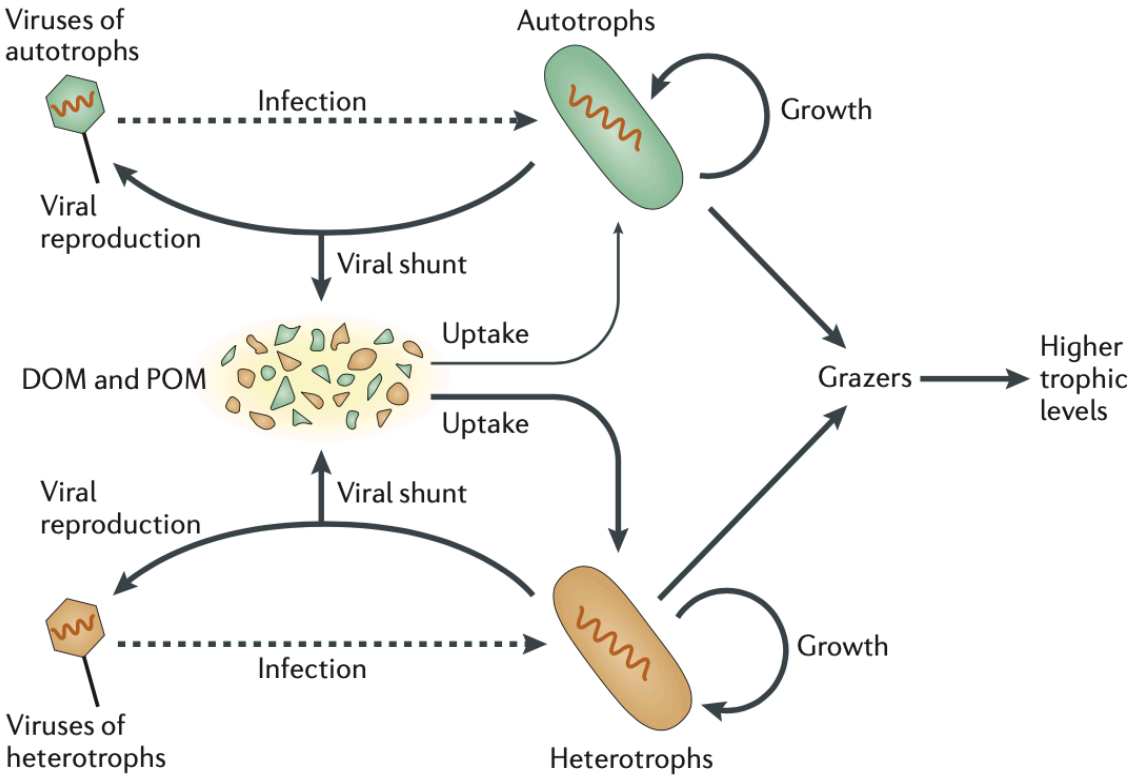


Figure 1: Virus-induced lysis of microbes releases cellular material into the environment, dissolved organic matter (DOM) and particulate organic matter (POM). This organic matter is returned into the microbial loop and prevents uptake by larger organisms in higher trophic levels. This process is called the viral shunt. **Figure acquired from (5).**

10. Southern Ocean microbes interact actively with present climate change and potentially influences the ocean's feedback on climate change

The Southern Ocean (SO) is the world's fourth largest ocean, and it connects the Pacific, Indian, and Atlantic oceans (235, 236). It is a unique circumpolar body of water that lacks continental barriers and encircles Antarctica completely (235-237). Surface temperatures in the SO range from -1.9°C to 8°C, depending on location and proximity to continental Antarctica (David and Saucède, 2015). Katabatic winds in the Southern Ocean drives a number of environmental conditions, including strong currents such as the Antarctic Circumpolar Current (ACC) (235, 238). The ACC is largest global water current and the major means of exchange of heat, salinity, and nutrients between

oceans (50, 239). As the ACC circulates, it transfers heat and CO_2 into the deep ocean (235, 240, 241). Therefore, the Southern Ocean contributes greatly to the global water circulation system and plays a pivotal role in climate regulation (237).

Oceanic fronts in the Southern Ocean

An oceanic front is a boundary between two distinct water masses (242, 243). With a water mass being defined as a body of ocean water that has a distinct range of salinity, temperature and a specific density that relates to the observed salinity and temperature (244). The Subtropical Front (STF) is a boundary that separates warm subtropical water from the cold Sub-Antarctic water (245). The STF flows in close proximity to the Agulhas Return Current (246). The STF runs eastward from 40°S in the Western Atlantic, below Africa's southern coast, through the Indian Ocean into the Eastern Pacific (245). South of the STF is the Subantarctic Front (SAF) (247). It is found at 48°–50°S in the Indian and Pacific Oceans, and at 42°–48°S in the Atlantic Ocean (248). Below the SAF is the Antarctic Polar Front (249). This front flows continuously around Antarctica and separates the Antarctic waters from the sub-Antarctic water (249). The cold, dense Antarctic surface water sinks rapidly below the Subantarctic water (248). As a result, the APF is characterized by fast currents as well as significant horizontal variations in density, temperature and salinity (250). The Southern Ocean remains one of the least studied oceans because of its remoteness and harsh climate (236). This lack of data has made it difficult to answer important questions about the Southern Ocean dynamics and microbial communities (251).

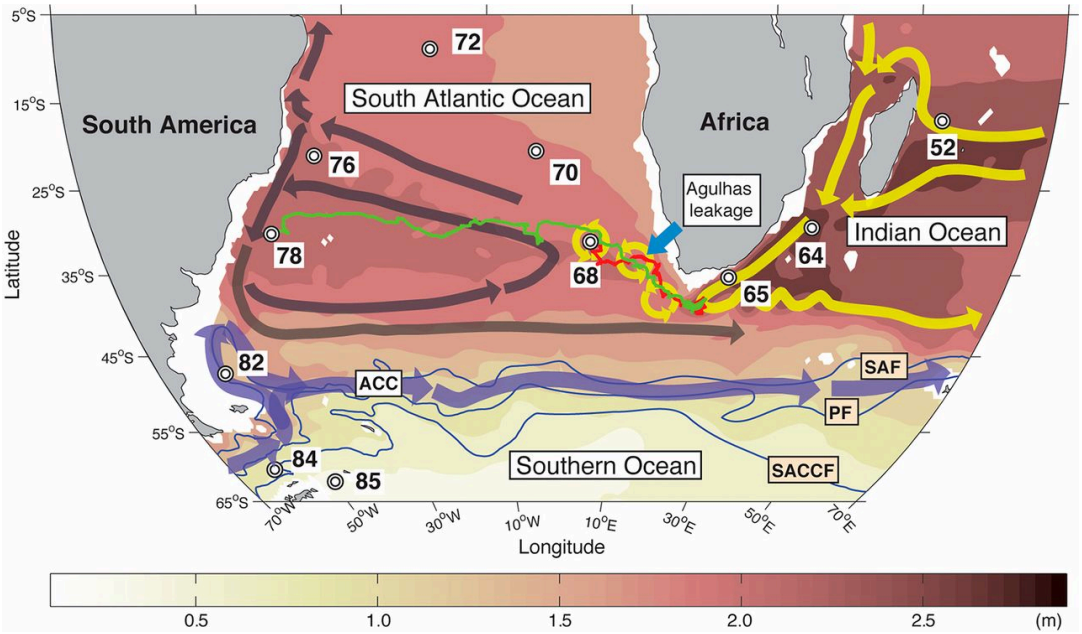


Figure 2. This image shows the Agulhas current, Agulhas rings, Agulhas retroflection and the and the different oceanic fronts associated with it. The ocean circulation is represented by arrows (currents) and different background colours [surface climatological dynamic height (0/2000 dbar from CARS2009; www.cmar.csiro.au/cars]. This picture was acquired from (252).

Features of the Agulhas Current potentially influence microbial diversity

Western boundary currents carry warm water from the tropics towards the poles, releasing heat into the atmosphere as the water cools (253-255). The Agulhas Current (AC) is a western boundary current on the east coast of South Africa (246). It forms near Mozambique and is primarily fed by the South West Indian Ocean sub-gyre (256), with additional source water from the Mozambique Channel (257, 258), and the East Madagascar Current (259). This current is the largest western boundary current in the world's oceans (260). The Agulhas Current transports warm, saline water and moisture, poleward from the Indian Ocean into the cold South Atlantic waters (261). It contributes to the moderation of rainfall and climate in Southern Africa by providing the latent heat of evaporation needed for onshore wind systems to carry moisture inland (46, 48, 262, 263).

The AC retroflects at the coast of South Africa, returning the waters to the Indian ocean in the Agulhas Return Current (ARC) (264). Some ARC-driven water leaks between

the South Indian and South Atlantic Oceans through the formation of large, warm, saline, anticyclonic eddies called Agulhas rings (262). The Agulhas rings have diameters ranging from 100-400 km, with up to six rings leaving from the Indian Ocean each year to the South Atlantic (265). Overall, this leakage, is a choke point for the distribution of heat, salt and biota across the Indian, Atlantic and Southern Oceans (266).

In the Agulhas Current, mass continuity, wind, and current forces drive surface water down, resulting in the upwelling of, nutrient-rich water from the deep ocean onto the surface (267). Upwelling of nutrients promotes primary activity (268-270). This is particularly evident in the waters of the Agulhas Retroflection, where chlorophyll-a concentrations are higher than the surrounding waters (271). Although these currents are known to affect biological production, little is known regarding their impacts on microbial structure and function, particularly viruses.

11. Problem statement

There is some recognition regarding the importance of microbial communities as mediators of ecosystem functions (203, 208, 272-274). The majority of studies have focused on characterizing bacterial communities and comparatively little is known regarding the role played by viruses (275-278). This knowledge deficit is particularly true for understudied microbiomes such as those in the South Indian and Southern Ocean (236, 279, 280). Given the central role of microbial communities as drivers of ecosystem functions, we urgently need to investigate the phylogeny and function of viruses in these oceans.

Previous studies have provided evidence showing that viral infection may drive microbial community structure and biogeochemical cycling (31-33, 35, 36, 214). Recent large-scale expeditions utilizing metagenomics, have expanded our understanding on the abundance, distribution, and biodiversity of viral communities in global oceans (56, 281, 282). However, we lack comparative insights into viruses in the Agulhas Current and Southern Ocean (268, 283).

To address this knowledge gap, this study assesses the diversity and potential biogeochemical function of viruses in the Agulhas Current and Southern Ocean. The upwelling of nutrients in the Agulhas Current positively influences primary production (270), which in turn may support the development of diverse microbial communities, including viruses (106, 284, 285). As a result, the viral communities in the Agulhas Current may exhibit higher diversity and potentially distinct functional characteristics, compared with those in the Southern Ocean (286). Our findings will provide a comprehensive virome data set in regions that have not been explored before. We will also provide insights into the phylogenetic diversity of viruses in the Agulhas Current and Southern Ocean. Analysis of AMGs in our viromes will provide insights into the functional diversity of viruses. Overall, studying viruses in these regions is important because marine viruses interact actively with present climate change, and influence the oceans' feedback on climate change (26).

12. Hypothesis

We hypothesize that the features of the Agulhas Current will influence the diversity and distribution of viral communities. The prevalence and diversity of viruses is predicted to be higher in the Agulhas regions compared to the Southern Ocean. We also predict that these shifts in phylogenetic diversity will translate to differences in viral function, through the expression of Auxiliary Metabolic Genes.

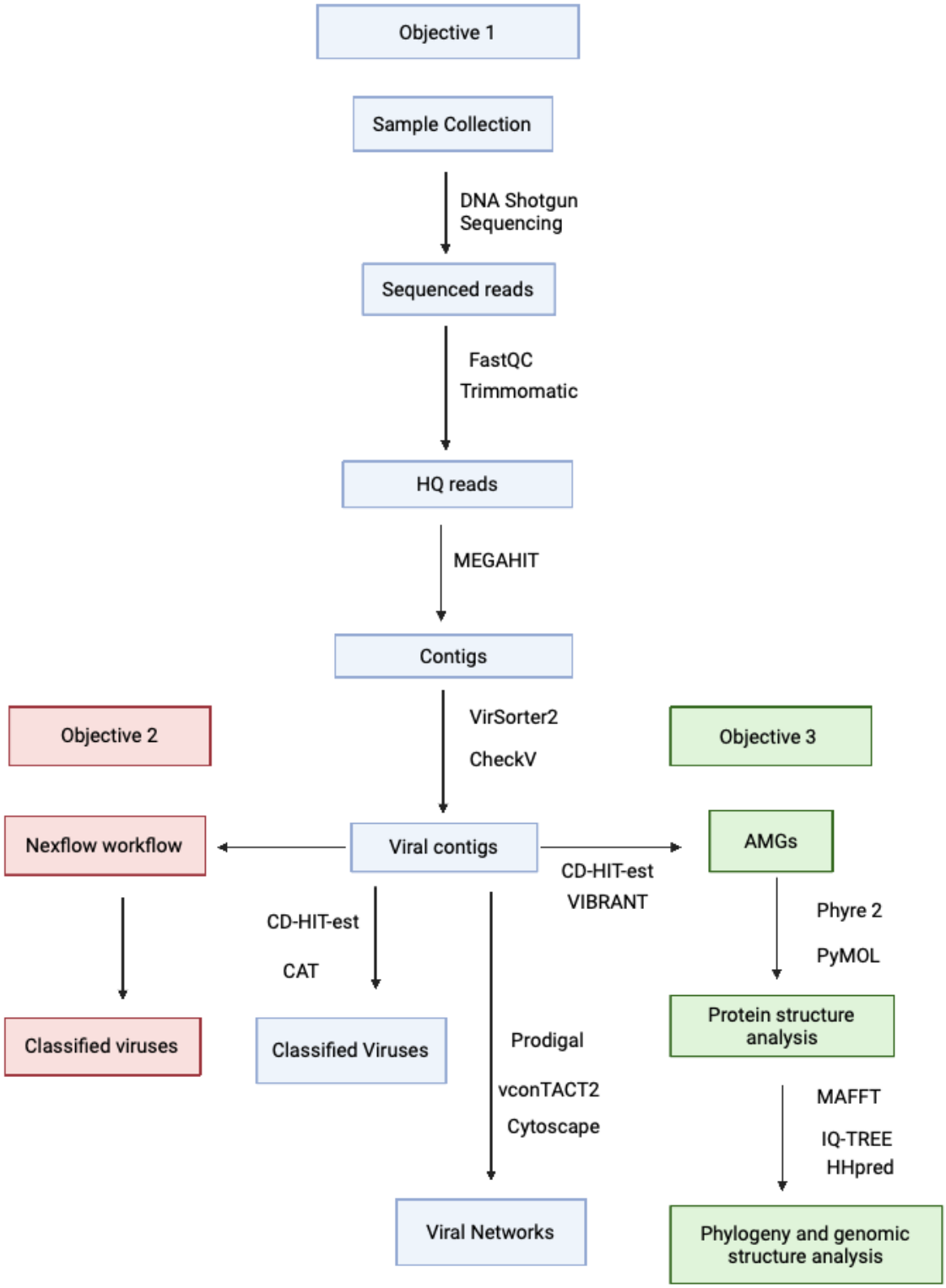
13. Aim

To assess the diversity and function of viral communities in the vicinity of the Agulhas Current and the Southern Ocean

14. Objectives

- To characterise the diversity of marine viruses in the Agulhas Current and the Southern Ocean
- To evaluate the effect of nutrient upwelling on the diversity and function of viral communities
- To investigate the biogeochemical influence of marine viruses in these regions, and their role in driving ecosystem functions

15. Flow Diagram



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17. Chapter 2

Methodology

Sample collection

Samples were collected during the South African National Antarctic Program (SANAP) 2022 Marion Island Relief Voyage. This cruise sailed from Cape Town to the Prince Edward Islands in the Southern Ocean (Figure 4). Samples were collected from the epipelagic zone (3 - 7 meters) of the ocean, using the underway and CTD system (Sea-Bird SBE-911 plus V2 CTD System; Sea-Bird Electronics, USA). The seawater samples collected (n=15) were size filtered using a peristatic pump (Masterflex VP pump, Milipore, USA). The initial filtration was done using 0.22 µm filter (Isopore Polycarbonate Membrane filter; Merck, South Africa) to exclude prokaryotes. Following this, 2 mL of 5% $FeCl_3$ was added to the filtrate and incubated for approximately 1 hour for viral flocculation as detailed previously (1). After incubation, samples were filtered through a 0.8 µm filter (Isopore Polycarbonate Membrane filter; Merck, South Africa) to collect the iron-precipitated viruses. The filters were stored at 4°C in sterile 5 mL cryotubes until further analysis.

DNA extractions and quality control

DNA was extracted from filters using the DNeasy PowerWater Kit (Qiagen, USA) with minor modifications to the protocol provided by the manufacturer. The filters were cut into small pieces, using a sterile scalpel under a laminar flow. This was followed by cell lysis, DNA binding, and column washing steps as per the manufacturer's instructions. However, the volume of the elution buffer was reduced to 50 µL to concentrate the DNA. The quality of the resultant DNA was evaluated, using a 0.8% agarose gel, and the concentration was determined using the Qubit dsDNA Assay Kit (Qubit 4 Fluorometer, Thermo Fisher Scientific, USA). For gel electrophoresis, 0.64 g Agarose was dissolved in 80 mL 1xTAE buffer and 1 µL SYBR Safe (Invitrogen, USA) was added. The gel was allowed to separate for 45 mins at 120 V and the DNA was visualised using a Gel doc system (Gel doc EZ, BioRad, USA).

Metagenomic sequencing

Samples (n=15) were sent for shotgun paired-end sequencing (2 x 150bp). The target output was 100 million (50 M x 2) reads (Admera Health, USA). The library was

prepared by Admera Health using the Nextera XT DNA library preparation kit (Illumina, USA).

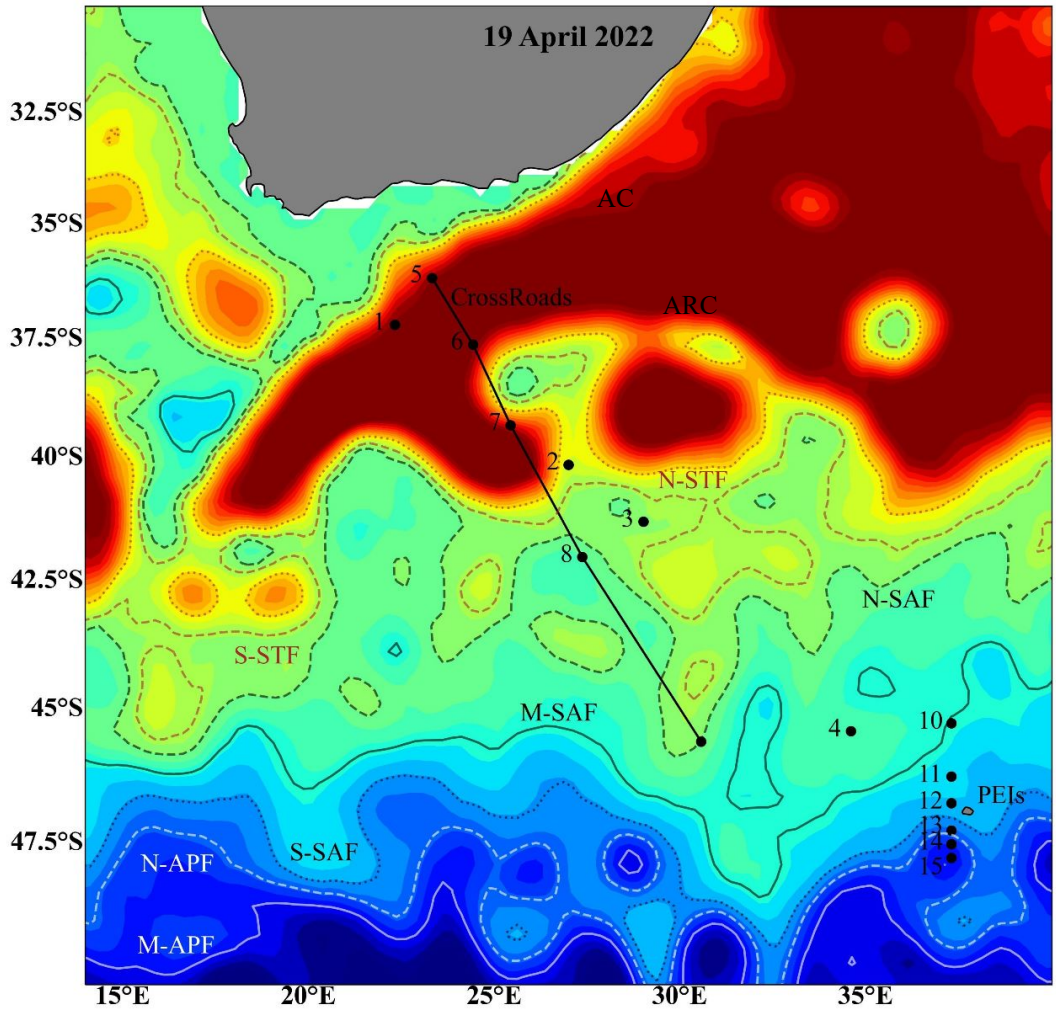

Figure 4. A map showing the Agulhas Current circulation, the distribution of samples (black bullets), the Southern Ocean and associated fronts. The Agulhas Current system was determined using sea surface heights from Maps of Absolute Mean Dynamic Topography (MADT, in meters)(2), with its position averaged over the duration of sampling. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **N-STF** – North Sub-Tropical Front, **S-STF** – South Sub-Tropical Front, **N-SAF** – North Sub-Antarctic Front, S**-SAF** – South Sub-Antarctic Front and **N-APF-** Antarctic Polar Front. **PEIs** – Prince Edward Islands. Image courtesy of Cristina Russo, University of Cape Town.

Bioinformatic analysis

Quality control and assembly

FastQC v0.12.0 was used to analyse the quality of the raw reads as detailed previously (3). The resultant reads were trimmed and filtered using Trimmomatic v0.39 (4). For trimming, low quality reads (<Q30 scores) and Nextera adapters were removed. The processed high quality reads were assembled using MEGAHIT v1.2.9 (5). The quality of the assembled contigs was analysed using Quast v5.2.0 (6). For all software, the default settings were used unless otherwise specified.

Identification of viruses

Contigs assembled from MEGAHIT were used to identify dsDNA viruses. Virsorter2 v2.2.3 (7) was used for this analysis using the following parameters: --include-groups dsDNAphage --min-length 5000 –min-score 0.5. A minimum length of 5,000 bp was selected and the resultant sequences were used for downstream viral classification. The minimum cut-off score, the “viralness score”, represents the likelihood that the input sequence is a dsDNA virus. CheckV v1.01 (8) was used to evaluate the quality of the identified viral contigs and to remove potential host regions**.** Virsorter2 v2.2.3 (7) was used to analyse the trimmed viruses generated from CheckV. The contigs were screened, based on the standard criteria as follows: viral_gene >=2 or viral_gene =0 and (host_gene =0 OR score >=0.95 OR hallmark >2) as previously described (https://www.protocols.io/view/viral-sequence-identification-sop-with-virsorter2-5qpvoyqebg4o/v3?step=4). Following this, CD-HIT-EST (9) was used to reduce redundancy from the viral contigs. The following parameters were used: --0.95 average nucleotide identity and --0.8 alignment coverage of sequence length. As a proxy for determining the distribution and relative abundances of dsDNA viruses across sampling sites, the CoverM version 0.6.1 (https://github.com/wwood/CoverM) tool was used to calculate transcripts per million (TPM) values.

Taxonomic assignments

The Contig Annotation Tool (CAT) (10) was used to assign taxonomy to the viral contigs using auto parameters. However, only a small fraction of the contigs were assigned taxonomy using this approach. Therefore, vConTACT2 v0.9.5 (11), using the default settings, was used to cluster viral contigs from our samples with reference viruses from the National Centre for Biotechnology Information (ProkaryoticViralRefSeq21). In addition to this, a custom nextflow workflow was

developed to assign taxonomy to non-redundant viral contigs from Virsorter 2 using blast (blastx, evalue 10^{-10} -outfmt -5). The script for the workflow is available on GitHub (https://github.com/nmafumo/Viral_diversity_workflow).

Auxiliary Metabolic Gene (AMG) identification and annotation

VIBRANT v1.2.1 (12) was used to identify the viral AMGs and metabolic pathways. Non-redundant viral contigs were used as input with **-**virome parameter. All AMGs identified by VIBRANT were visualized using TBtools (13). AMGs of interest, including sulfur metabolising genes, were manually curated and visualised using the ggalluvial package in R v4.1.1 (14).

Protein structure predictions, genetic architecture plots and phylogenetic analysis of aprB genes

Phyre2 v 2.1 (15) was used to predict the structure of three sulfur metabolism AMGs. We specifically characterized *apr*B genes which were not previously identified in viruses. These analysis bolstered the annotations avoiding erroneous characterizations based solely on sequence similarity searches. Following this, genetic architecture plots were used to visualize the organization of genes on three contigs with *apr*B genes**.** HHpred version v2.08 (16) was to annotate proteins in these three contigs using the following parameters: --B 10 --Z 10. The gggenes package in R (https://wilkox.org/gggenes/) was used to visualise genetic architecture plots**.**

Maximum likelihood phylogenetic analysis was performed to determine the evolution of viral *aprB* gene. AprB sequences from our data were queried against the RefSeq database to identify closely related viral and bacterial sequences. Following this, sequences were aligned using MAFFT with the -auto parameter (17). The resultant alignment file from MAFFT was used to reconstruct a maximum likelihood tree with 1,000 bootstraps with IQ-TREE (18). The resulting trees and bootstrapping values were visualized with the Interactive Tree of Life v6 (iTOL) (19).

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18. Chapter 3

The diversity of viruses in the Agulhas Current and Southern Ocean

Abstract

Microbial communities are essential for the function of ocean ecosystems through their contributions to biogeochemical recycling. Viruses play a pivotal role as core members of these communities, and actively influence the abundance, diversity, and evolution of their cellular hosts. Thanks in part to several large scale marine expeditions (including the Tara Oceans, Malaspina, Pacific Ocean Virome), we are beginning to understand the distribution and biodiversity of viruses in most global oceans. However, we lack comparative insights of viruses in the Agulhas Current and Southern Ocean, both of which are important and critical regions for mitigating the effects of climate change. To reduce this knowledge deficit, we provide a data set of 47 698 dsDNA viral operational taxonomic units (vOTUs) from the Agulhas Current, Agulhas Return Current and oceanic fronts of the Southern Ocean. Most of the viral contigs (64%) found in this study were unclassified, and these sequences failed to match known viral taxa. However, of the validly classified viruses, Caudovirales dominated both the warm Agulhas and cold Southern Ocean water masses. *Synechococcus* and *Prochlorococcus* viruses were highly prevalent in warm waters but completely absent in the Polar Fronts of the Southern Ocean. Viruses of other dominant bacterial heterotrophs including *Pelagibacterales* (SAR11) and *Puniceispirillum* (SAR116) were also found. The results of this study suggest that viruses play a crucial role in shaping microbial communities of major primary producers in the Agulhas Current. This has important implications for our understanding of the role of viruses in marine ecosystems, and how they may respond to climate change.

Introduction

Viruses are highly abundant and diverse constituents of marine ecosystems (1-4). They play a key role in shaping microbial communities through horizontal gene transfer and viral-mediated mortality (5-10). Additionally, viruses contribute to microbial biogeochemical cycles through viral lysis and metabolic reprograming of the host (11, 12). Notably, viral lysis releases dissolved organic carbon (DOC) into the environment, preventing its flow to higher trophic levels (13-15). Cell components of prokaryotes released by viral lysis are a major source of recalcitrant dissolved organic matter in the microbial carbon pump, a model of long-term carbon storage in the global ocean (16). Hence, viruses are key mediators of ocean carbon sequestration through the microbial carbon pump and biological pump (16, 17). Despite their importance, most studies of viral distribution and function have been conducted in the Pacific, Indian and Atlantic Oceans (18-23), with relatively few studies in the Southern Oceans (24, 25). Addressing this knowledge gap will contribute to our understanding of the global role of viruses in marine ecosystems.

Large-scale metagenomic studies of marine viruses have brought significant advances to our understanding of viral genomic diversity in global oceans (20, 22, 23). These studies have revealed an unexpected diversity of novel viruses in both surface and deep waters (18, 20, 23, 26-29). In the surface ocean, dsDNA viruses are known to infect dominant microbial groups including picocyanobacteria like *Prochlorococcus* and *Synechococcus,* (9, 30-32) and heterotrophs such as *Pelagibacterales* (SAR11) and *Puniceispirillum* (SAR116) (33-36). Indeed, across the Pacific, Indian and Atlantic Oceans viral abundance has been shown to increase with increasing chlorophyll a concentration and photoautotrophic picoplankton abundance (37, 38). The high abundance of phytoplankton provides a large pool of potential hosts for viruses (37). These findings suggests that the availability of phytoplankton hosts is a major driver of viral dynamics in the marine environment (39). This relationship has important implications for understanding the role of viruses in the marine carbon cycle, as viruses can divert carbon from phytoplankton to other microbes through the viral shunt (40).

The Agulhas Current (AC) is the largest western boundary current in the world's oceans (41). This current is distinguished by high wind speeds, which contribute to the moderation of rainfall and climate in Southern Africa (42). The Southern Ocean (SO) is below the AC, and is one of the largest oceanic carbon sinks (43). The cold temperatures in the SO contribute to a relatively high solubility of CO_2 (44, 45). The extensive growth of phytoplankton blooms in this region results in the absorption of substantial quantities of CO_2 for photosynthesis (46, 47). Despite recent advances in phytoplankton and physicochemical flux studies in this region (48-50), we still lack a comprehensive understanding of microbial phylogeny and function, including viruses (51). Given the central role of the Agulhas Current and the Southern Ocean in the global climate system, we urgently need to study microbial communities in these regions. Based on previous studies, it is reasonable to predict that viral communities substantially contribute to mediating carbon and nutrient biogeochemical cycling in this ocean (24, 52).

To reduce the current knowledge deficit, we characterise viruses within the vicinity of the Agulhas Current and the Southern Ocean. We predict that the upwelling of nutrients in the Agulhas Current, caused by the high speed of the current and wind, may increase the prevalence of viral communities in this region (52, 53). Based on previous studies that have shown the effects of water mass endemicity and the structural determinants on bacteria, we further predict that the distribution of viruses may vary based on the specific oceanographic water masse (54). In line with these predictions, using relative abundance estimates, we observed a high prevalence of dsDNA viruses in the warm waters of the Agulhas regions. For example, certain species including *Synechococcus* and *Prochlorococcus* viruses were completely absent in the Sub-Antarctic and Polar Fronts of the Southern Ocean. Furthermore, viruses of *Pelagibacterales* (SAR11) and *Puniceispirillum* (SAR116) were highly represented in our metagenomes, suggesting that they potentially play prominent roles in these marine ecosystems.

In this study, we also designed an automated nextflow workflow to assign taxonomy to viral Contigs from Virsorter2. This workflow is adaptable, and may be curated to include different reference databases. The workflow can be integration with other taxonomic classification methods to enhance the robustness of viral taxonomic

assignments. Taken together, our results suggest that viruses in the Agulhas Current and Southern Ocean potentially influence carbon recycling through viral lysis. A better understanding of the viral shunt and the metabolic processes orchestrated by viruses in these regions could provide valuable insights into the precise dynamics of the ecosystem, including microbial interactions and biogeochemical cycling.

Results

Novel viral genomes are enriched in the Agulhas Current and Southern Ocean

In total, 79 933 viral contigs were retrieved by analysing epipelagic samples from the Agulhas Current and the Southern Ocean. These contigs were clustered into 47 698 dsDNA viral operational taxonomic units (vOTUs). Of these non-redundant viral contigs, approximately 64% (30 525) constituted ‘novel populations’ which were not represented in publicly available databases. Approximately 7% (3358) of the vOTUs were classified at the class level (Figure 5a) and only 5% (2606) were classified to the family level, using read alignment comparisons with the Contig Annotation Tool (CAT) (55).

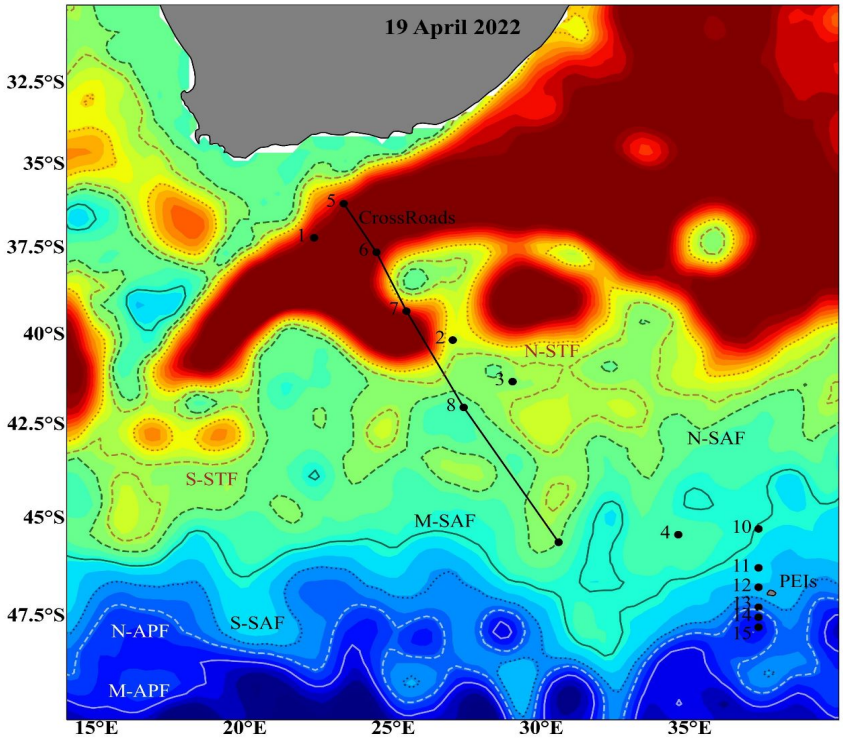

Figure 5. A map showing the Agulhas Current circulation, the distribution of samples (black bullets), the Southern Ocean and its associated fronts. The Agulhas Current system was determined using sea surface heights from Maps of Absolute Mean Dynamic Topography (MADT, in meters)(56), with its position averaged over the duration of sampling. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **N-STF** – North Sub-Tropical Front, **S-STF** – South Sub-Tropical Front, **N-SAF** – North Sub-Antarctic Front, **S-SAF** – South Sub-Antarctic Front and **N-APF-** Antarctic Polar Front. Image courtesy of Cristina Russo, University of Cape Town.

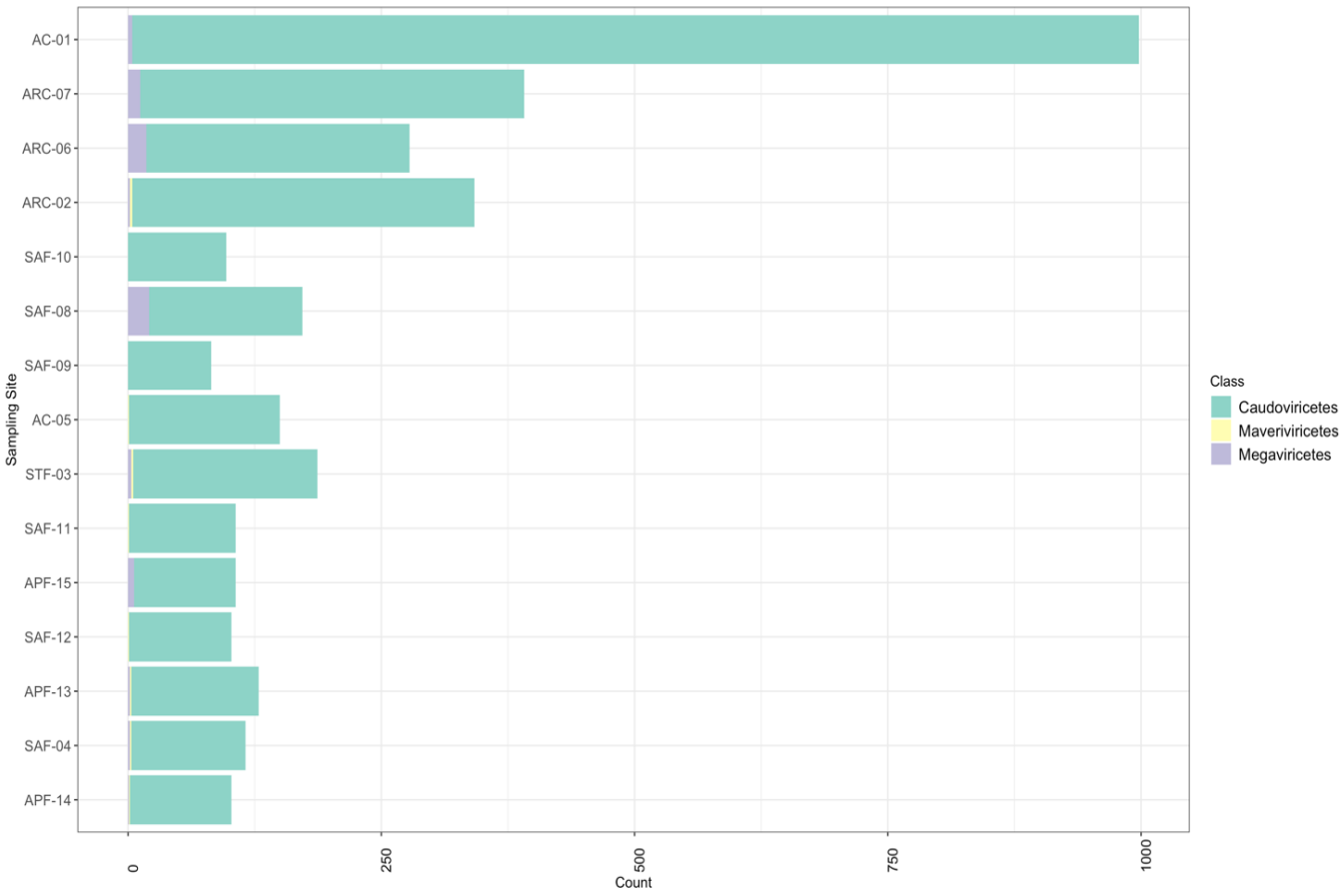

Figure 5a. The distribution of viruses (at class level) across the different sampling sites. The counts were obtained using the Contig Annotation Tool (CAT). Caudoviricetes were highly prevalent at all sampling sites. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **STF** -Sub-Tropical Front, SAF - Sub-Antarctic Front and **PF-**Polar Front.

Protein network clustering of viral families reveals that photoautotrophic picoplankton dominates these regions

Due to low level of positive classifications obtained from reference-based assignments, we assigned taxonomy using viral clustering methods. Of the vOTUs, 36% were assigned taxonomy and Caudoviricetes were found to be the most dominant class (Figure 5b). Of these Caudoviricetes phages, *Synechococcus* (51%), *Prochlorococcus* (21%), Cyanophages (12%) and Pelagiphages (4%) were found at higher abundances (Figure 6a, 6b).

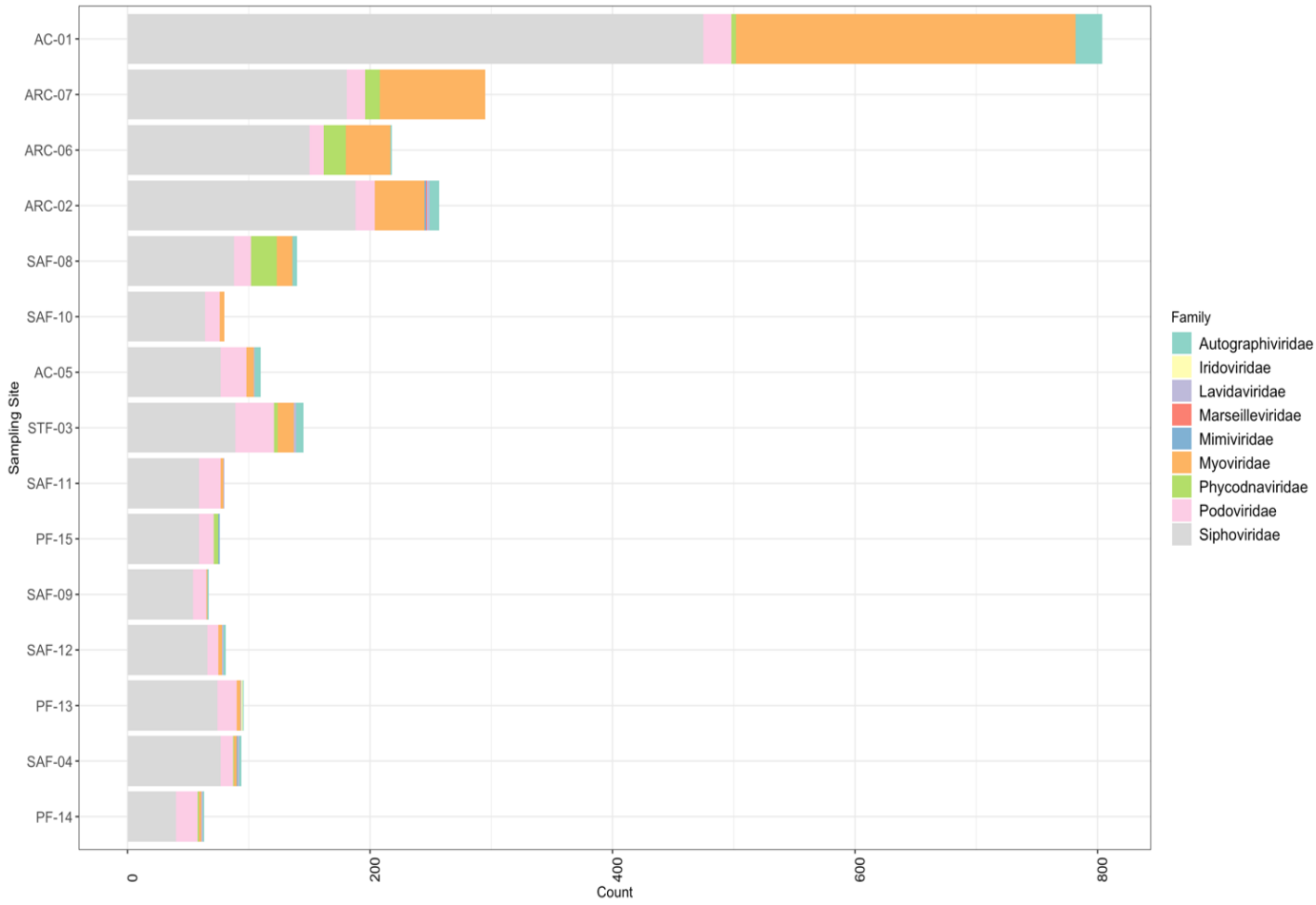

Figure 5b. The distribution of viral families across the different sampling sites. The counts were obtained using the Contig Annotation Tool (CAT). Myoviridae, Podoviridae and Siphoviridae are the most prevent taxa in all the sampling sites. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **STF** -Sub-Tropical Front, SAF - Sub-Antarctic Front and **PF-**Polar Front.

Viral identification using Nextflow

In this study, we developed a Nextflow analyses workflow to automate the taxonomic assignment of non-redundant viral contigs identified using Virsorter2. This workflow leverages BLAST, in conjunction with a user-selected database, for taxonomic assignments. In addition, the workflow creates a working directory for the resultant data. Following the parsing of BLAST results, the output includes a structured text file with columns displaying important attributes of the similarity search including information on the contigID, associated phage sequence ID, alignment length, e-value

and corresponding bitscore. The results from example analysis can be found here https://github.com/nmafumo/Viral_diversity_workflow.

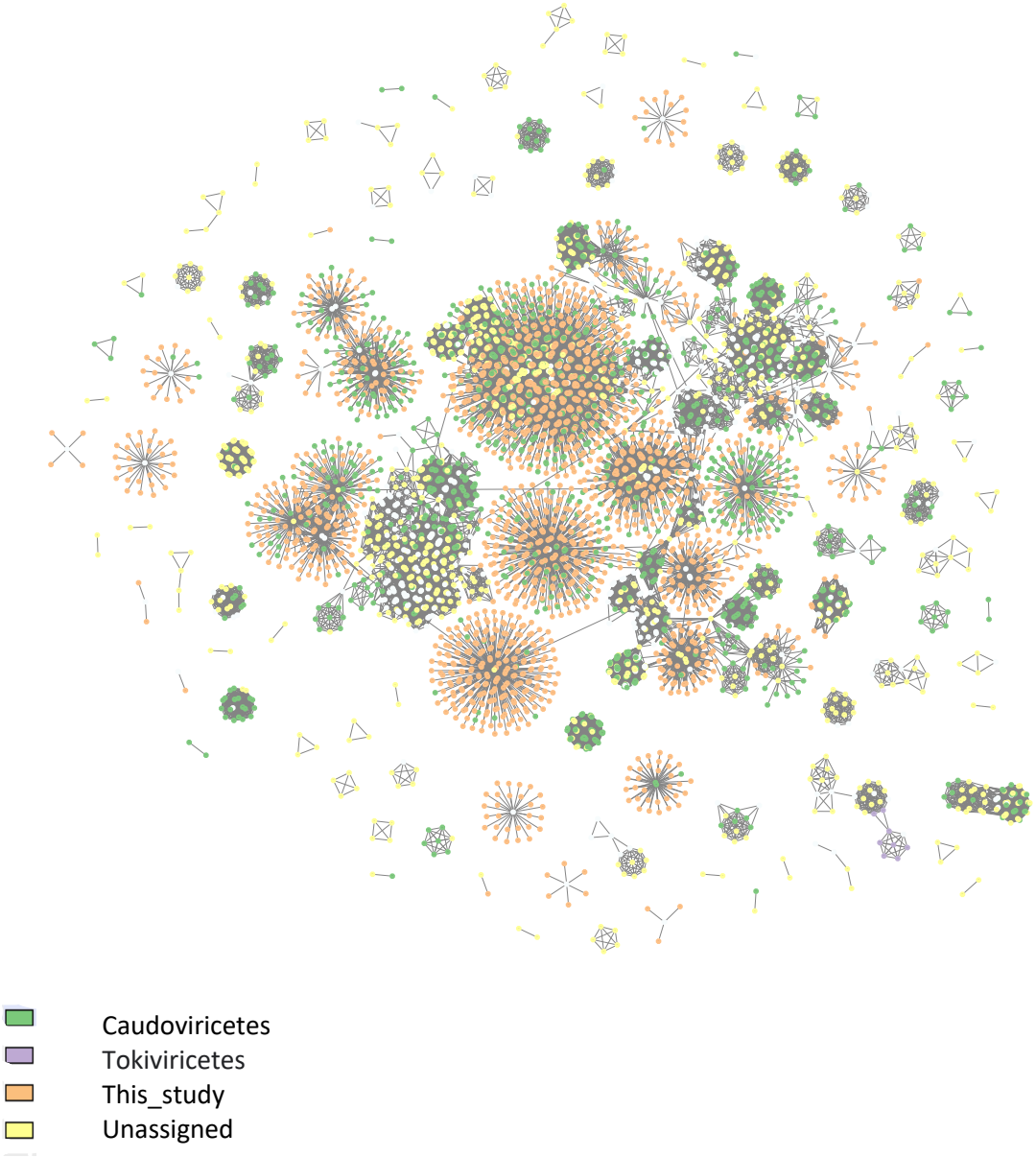

Figure 6a. Protein network clustering networks showing viral contigs obtained in this study and sequences from a reference database. In the protein network, each shape represents a single viral population or reference phage. Shapes are connected by lines respective to shared protein content. Viral population taxonomy was coloured according to the order. Caudovirales order is most abundant.

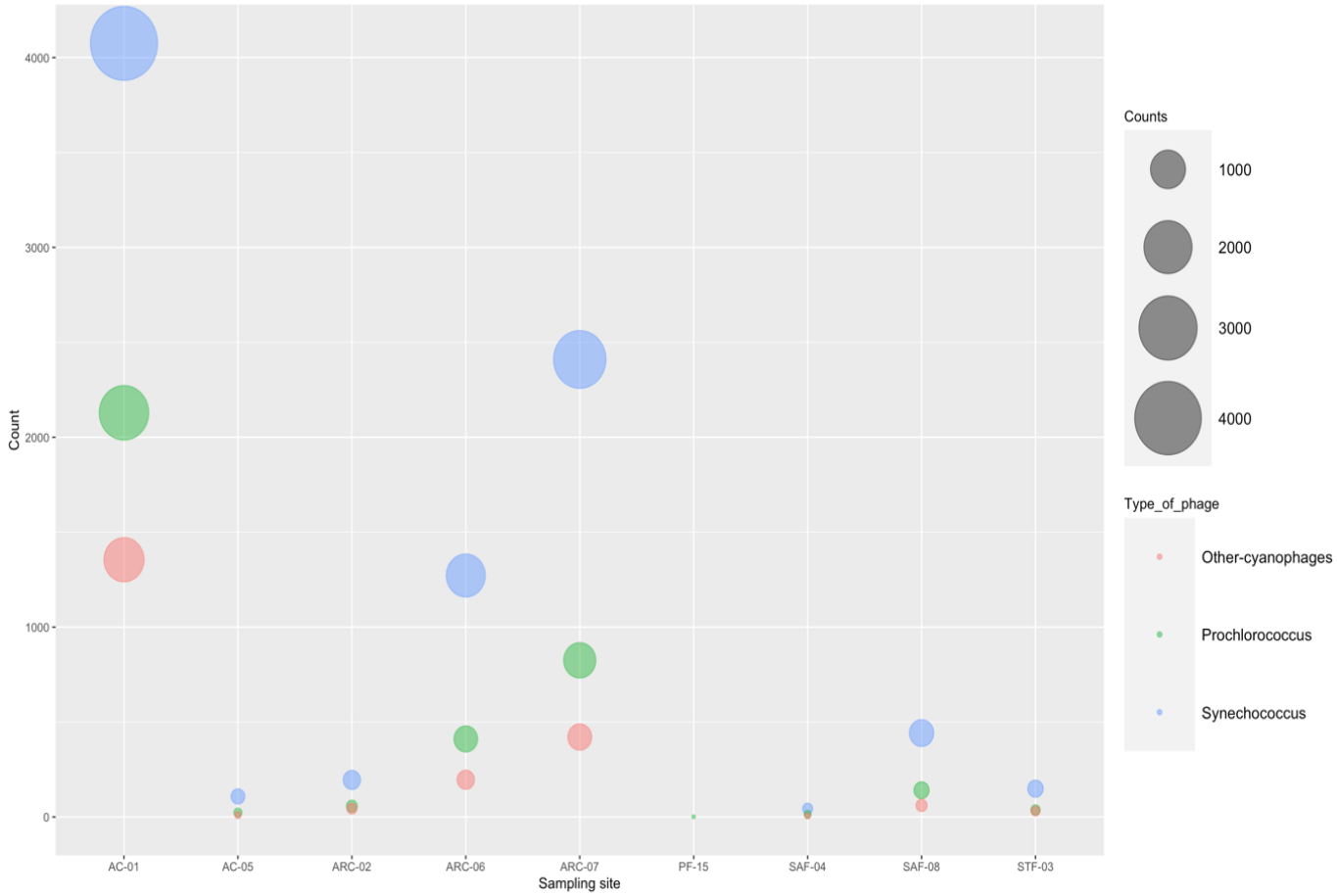

Figure 6b. The distribution of the top 1 % most abundant bacteriophages obtained from the different sampling sites using network clustering. Synechococcus and *Prochlorococcus* were highly prevent in the warm waters of the Agulhas regions. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **STF** -Sub-Tropical Front, SAF - Sub-Antarctic Front and **PF-**Polar Front.

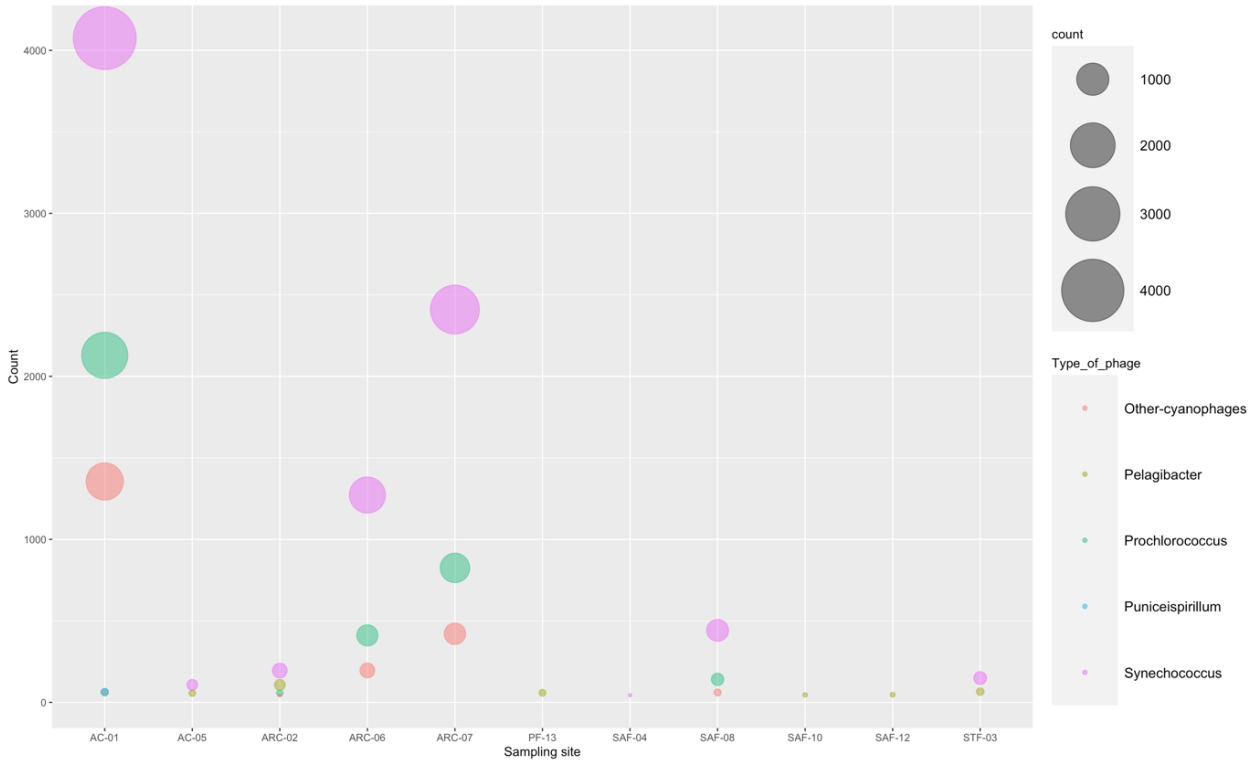

Figure 6c. Distribution of the top 0.25 % bacteriophages obtained different sampling sites using network clustering. Synechococcus and *Prochlorococcus* are highly prevent in the warm waters of the Agulhas regions. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **STF** -Sub-Tropical Front, SAF - Sub-Antarctic Front and **PF-**Polar Front.

Discussion

In this study, we provide a comprehensive dataset of 79 933 viral genomes in the Agulhas Current and the Southern Ocean. These oceans represent two underexplored regions of the global ocean, and our findings provide essential baseline data on viral communities. Consistent with our hypothesis, we observed more viruses in the Agulhas Current compared to the Southern Ocean (Supplementary Table 4). This is because warmer regions such as the Agulhas Current system, tend to have higher relative abundances of viruses as these conditions favour the growth of phytoplankton host cells (54, 57, 58). Furthermore, the upwelling of nutrients in the Agulhas Current (53) promotes primary production (59). Areas of high primary production have been linked to high viral abundance in the euphotic zone (60). Therefore, viruses in the Agulhas Current and Southern Ocean potentially play an important role in regulating phytoplankton populations in these regions.

Cyanophages are a major component of the viral community in the epipelagic ocean (20, 23, 61). Cyanophage abundance has been shown to correlate with the abundance of their photoautotrophic hosts (62-64). In the central Pacific and Southern Ocean, 57% of the total virioplankton abundance were affiliated with *Prochlorococcus, Synechococcus and* picoeukaryotes (60). Our results align with this finding, as cyanophages were the most ubiquitous class recovered in our viromes. Cyanophages carry many functional genes of their hosts which allow them to effectively proliferate in oligotrophic oceanic environments (8, 65-68). However, the decline or absence of *Prochlorococcus* viruses, in the Antarctic Polar Front and regions that are close to this front, may be explained by the thermal limits of *Prochlorococcus* growth. *Prochlorococcus* have been shown previously to be absent in waters with temperatures below 15^{o} C (69). Similarly, the response to temperature decline, as observed in *Synechococcus*, aligns with previous marine work that indicates a decrease in *Synechococcus* abundance as temperatures decrease (62).

The dominance of cyanophages in the Agulhas Current and the Southern Ocean has important implications for biogeochemical cycles (68, 70-72). When cyanobacteria are lysed by viruses, the fixed carbon they contain is not used for growth and biomass accumulation; instead, it is released back into the environment as dissolved organic matter (13). This redirects the flow of matter and energy from higher trophic levels (14). Although viruses release a small quantity of carbon in the deep sea, there is some evidence that this carbon is more biologically available (4, 73). This available carbon source has the potential to alleviate organic carbon limitation for heterotrophic prokaryotes, thereby serving as a source of energy to drive prokaryotic activity (74, 75). Additionally, viral lysis releases essential nutrients that limit primary production, such as nitrogen, phosphorus, and iron, back into the environment (15, 74, 76). These findings align with previous work in the Pacific Ocean, which demonstrated that viral lysis may be a significant pathway in carbon cycling at the epipelagic ocean (29).

Our results suggest that viral infection and lysis may affect the efficiency of the biological pump (16, 17), in the Agulhas Current and Southern Ocean**.** This could ultimately affect the proportion of carbon that is sequestered in the deep sea (2, 16)**.** However, there are still several open questions about how viral lysis affects the biological pump. Some studies suggest that viral lysis reduces the efficiency of the

biological pump by releasing nutrients back into dissolved organic matter (77). Other studies suggest that viral lysis increases the efficiency of the biological pump by accelerating the export of host organisms from the euphotic zone (78, 79). Moreover, other studies suggest that viral lysis facilitates particle aggregation and the transfer of carbon into the deep sea by releasing adhesive colloidal cellular components (80-82). Future research on viral ecology is essential for understanding and predicting the response of marine ecosystems to climate change.

The widespread alphaproteobacterial group *Pelagibacterales* (SAR11 clade) dominates the global oceans, potentially comprising as much as one-third of all marine microbial communities (83, 84). Viruses of the SAR11 members, often referred to as pelagiphages, are the most abundant phages in the global oceans (85, 86). Indeed viruses of *Pelagibacterales* (SAR11) and *Puniceispirillum* (SAR116) dominated our metagenomes, which is consistent with results from the Indian and Pacific Ocean viromes (35, 36). The SAR11 and SAR116 clades are groups of bacteria that play important roles in the global sulfur cycle. They contain genes that allow them to produce dimethylsulfide and degrade dimethylsulfoniopropionate, two important sulfur compounds (87, 88). Their phages, could indirectly influence the sulfur cycle by infecting and lysing these bacteria. Furthermore, the dominance of pelagiphages in our viromes implies that they may have a significant role in regulating SAR11 population dynamics which influences global carbon cycling (89). Despite significant cell loss of SAR11 populations due to viral predation, their high recombination frequencies allow for the rapid evolution of phage-defence alleles, maintaining a high abundance of both hosts and phages (85). This coevolutionary dynamic, demonstrates the Red Queen Hypothesis (90), where SAR11 is both abundant and susceptible to phage predation in these regions.

Conclusion

Taken together, the results of this study provide highlight the extent of diversity and distribution of dsDNA viruses in the Agulhas Current and Southern Ocean. We provide a comprehensive data set of 47 698 (vOTUs) in regions that have not been explored before. However, a substantial proportion of the vOTUs remains unclassified, limiting

our knowledge of their taxonomy and ecological roles in these regions. The high abundance of the SAR11 clade and Cyanobacteria in surface seawater, coupled with our finding that these groups were also highly represented in the viral community, suggests that higher occurrence of bacterial hosts results in a higher prevalence of the phages that infect them. The prevalence of phage groups targeting *Synechococcus, Prochlorococcus*, *Pelagibacterales* and *Puniceispirillum* highlights the potential role of viruses in influencing energy and carbon cycling through the viral shunt**.** Overall, our results suggest that viruses play a crucial role in shaping the ecological succession of major marine primary producers and the cycling of organic matter in the Agulhas Current**.** We are only beginning to understand viral taxonomy and ecology in these regions. Future research is needed to expand reference databases and to develop methods that can help us better understand viral communities, viral-host dynamics, and their biogeochemical influence.

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19. Chapter 4

Viruses as drivers of sulfur cycling in the Agulhas Current and Southern Oceans

Abstract

Auxiliary metabolic genes (AMGs) have been shown to mediate important biogeochemical cycles in terrestrial and marine environments. In the oceans, marine viruses appear to substantially modulate the metabolism of their hosts. However, previous studies have focused on the evolution and ecological function of AMGs in the Pacific, Atlantic and Indian oceans. This has left significant gaps in our understanding of viral contributions to the ecology of most marine regions, particularly in their potential role in mitigating the effects of climate change. For instance, marine regions such as the Agulhas Current System and the Southern Ocean play critical roles in regional and global climate circulation, yet the influence of viruses in these ecosystems remains unclear. Here, we assess viral diversity, distribution, and potential function of AMGs in these regions to elucidate putative ecological roles. In total, 5656 viral AMGs were annotated and classified into 260 KEGG orthologous groups. Functional annotation suggests that the majority of these AMGs are essential for biogeochemical recycling, with a large proportion involved in carbon (~36%) and vitamin/cofactor (~31%) metabolism. Of the AMGs linked to energy metabolism, a high proportion of identified genes were implicated in sulfur metabolism compared with those linked to photosynthesis. These genes include *fcc*A, *sox*Y, *dsr*C, *dsr*A and *apr*B, which are known to play important roles in sulfur and thiosulfate oxidation. Overall, our dataset suggests that viruses in the Agulhas Current and Southern Ocean harbour diverse mechanisms, which have potential to influence the biogeochemical cycling of sulfur through the expression of AMG. Given that approximately half of marine microbes may be infected by viruses at any given time, viral contributions to nutrient recycling may be substantial. The results of this study provide important baseline data required for the integration of viral community dynamics in Earth system models.

Introduction

Marine viruses constitute the primary source of prokaryotic mortality, with estimates suggesting that viral infections may be in the region of 10^{28} infections per day (1). These viral infections considerably affect microbial diversity, and related biogeochemical cycling, through alterations to host metabolism and the expression of auxiliary metabolic genes (2-4). Viral AMGs are horizontally acquired genes, which are introduced into the viral genome during infection (5-7). These genes are highly similar to the host homologs, encode for proteins which perform the same metabolic functions those of the host cell (8, 9). However, the functional role of AMGs and their contributions to host metabolism and biogeochemical cycling remains largely unclear (10).

Current studies suggest that AMGs may significantly affect global biogeochemistry and the evolutionary dynamics of microbial metabolism (5, 11-14). Estimates suggest that up to 40% of microorganisms, known to be the primary drivers of biogeochemical cycles in these oligotrophic systems, may be infected by viruses at any given time (1, 15). These viral infections have a substantial influence on the activities of these microorganisms and the ecosystem services they provide, such as organic matter recycling throughout the water column (16, 17). Emerging evidence suggests that viral AMGs play a significant role in global biogeochemistry (8, 18-20). Studies on cyanophages have shown that AMGs may play a role in a diverse assortment of metabolic functions including so called "phage photosynthesis" (21, 22). The presence of photosynthetic genes in cyanophages is suggested to increase phage fitness by supplementing energy production during infection (19, 23, 24). Moreover, cyanophage genomes may harbour over 20 AMGs, which may modify the electron transport chain and strengthen carbon metabolism in their hosts (8, 19, 20, 25). In addition to carbon metabolism, AMGs influence the metabolism of other nutrients including phosphate, sulfur, and nitrogen (14, 26-29). These results suggest that AMGs may play a vital role in the biology and ecology of cyanophages by conferring a wide range of metabolic functions. While several studies have shown the importance of AMGs linked to carbon (8, 23, 26, 30), phosphorus (12, 31) and nitrogen (32-34) metabolism, comparatively less is known regarding the role played by viruses in sulphur recycling (11, 35).

Dissimilatory sulfur metabolism (DSM) plays a crucial role in biogeochemical processes (36). The taxa implicated in DSM span across diverse phyla and include a suite of microorganisms with capacity to use sulfur compounds for generating energy and for critical metabolic reactions (11, 36). While the role of viruses in DSM is poorly understood, several bacteriophages that encode genes for sulfur metabolism have recently been identified (11, 34, 35, 37). These studies suggest that bacteriophages with the capacity to encode sulfur metabolism, may be crucial determinants on the rates of DSM in their hosts (29, 35). There is some evidence that these bacteriophages directly contribute in the fluxes and budgets of sulfur within the community (35, 38). However, despite growing recognition of their importance, our understanding of sulfur-metabolising viruses and their interactions with their hosts remains limited.

Here, we investigated viral contributions to biogeochemical cycling in the Agulhas Current and the Southern Ocean. In addition to providing novel insights on several well characterized metabolisms, we provide detailed results regarding phage-directed sulfur metabolism. The results suggest that that the AMGs identified in this study may improve the ecological fitness of viruses and consequently argument host metabolic processes. Of the sulfur metabolisms identified, *dsr*A*, dsr*C*,* and *sox* genes are known to enhance key steps in host mediated sulfur metabolism during infection (29, 39). Taken together, our results suggest that viruses play a significant role in the biogeochemical cycling of sulfur in the Agulhas Current and the Southern Ocean. The results of this study substantially advance our understanding of the phylogenetic repertoire AMGs in the Agulhas current system, which improves our knowledge of viral ecology and evolution in this region.

Results

Auxiliary metabolic genes involved in energy and vitamin metabolism dominate the Agulhas Current and the Southern Ocean

In total, 5656 viral AMGs were annotated and classified into 260 KEGG orthologous groups from 47 698 vOTUs (Supplementary Table 1). The majority of these AMGs (2253) were from samples retrieved from locations in the vicinity of the Agulhas

Current (5 sites). The remaining AMGs were distributed among the other 10 sampling sites. The AMGs obtained from the Agulhas Current formed discrete clusters from those obtained from the Southern Ocean (Figure 7). Furthermore, the most abundant AMGs in our dataset were those found to encode a suite of functions which were previously shown to protect viruses from degradation by their hosts (Supplementary Table 1). For instance, DNA (cytosine-5)-methyltransferase 1 (DNMT1) constituted roughly 10% of the total AMGs identified in the study.

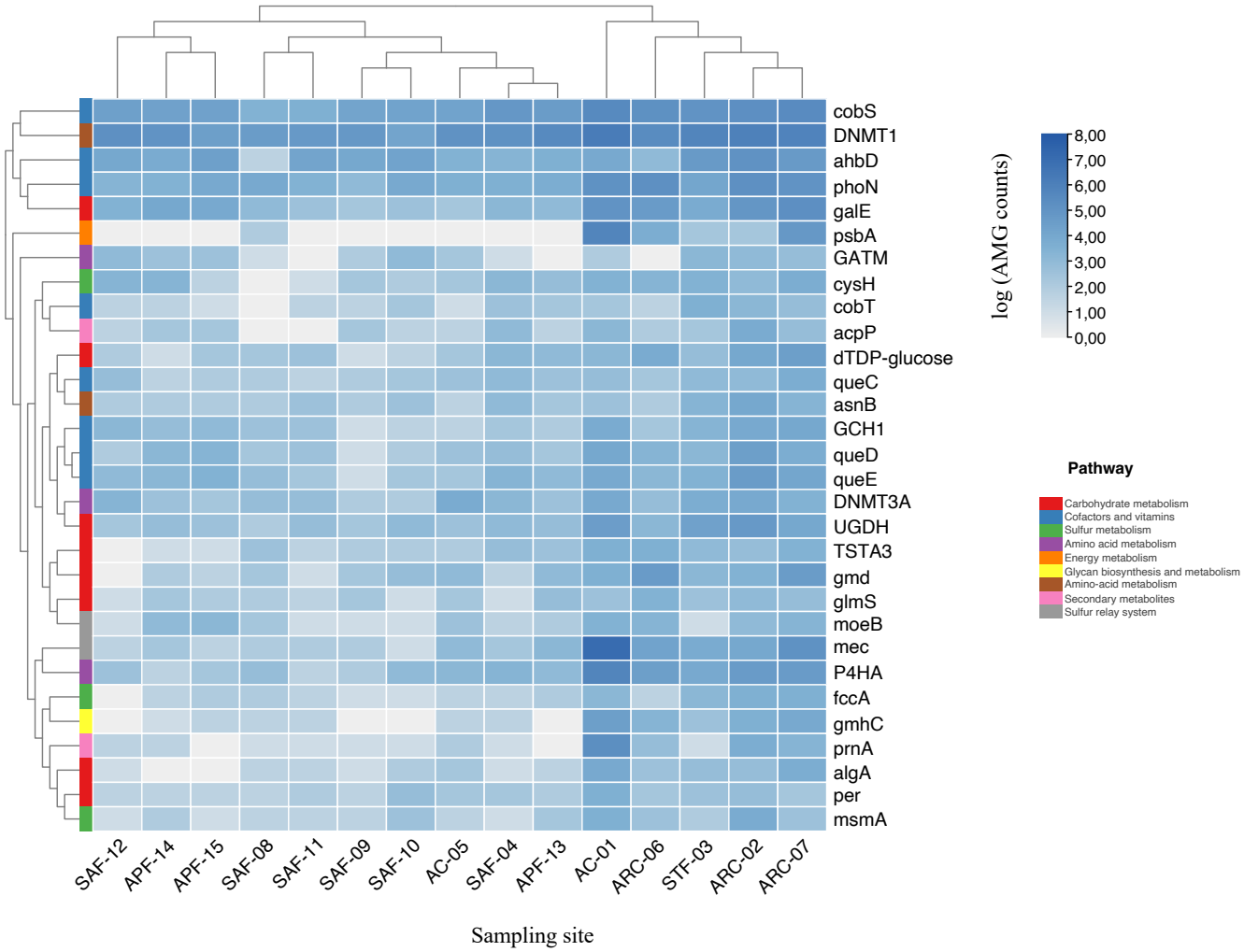


Figure 7. A heatmap showing the top 1% of AMGs recovered from our dataset and the respective associated pathways. The analyses showed that more AMGs were sourced from the Agulhas Current and the Agulhas Return Current compared with regions in the Southern Ocean. Furthermore, AMGs from the Southern Ocean and those from the Agulhas regions formed distinct clusters. **AC**- Agulhas Current, **ARC**- Agulhas Return Current, **STF** -Sub-Tropical Front, SAF - Sub-Antarctic Front and **PF-**Polar Front.

Other prevalent AMGs, identified in this study, include genes associated with vitamin/co-factor (32%) (Figure 8) and energy metabolism (23%) (Figure 9). From the

AMGs involved in energy metabolism, more genes were involved in sulfur metabolism (19%) compared with those involved in photosynthesis (11%). These AMGs were linked with *Siphoviridae* and *Myoviridae* genomes (Supplementary Table 3).

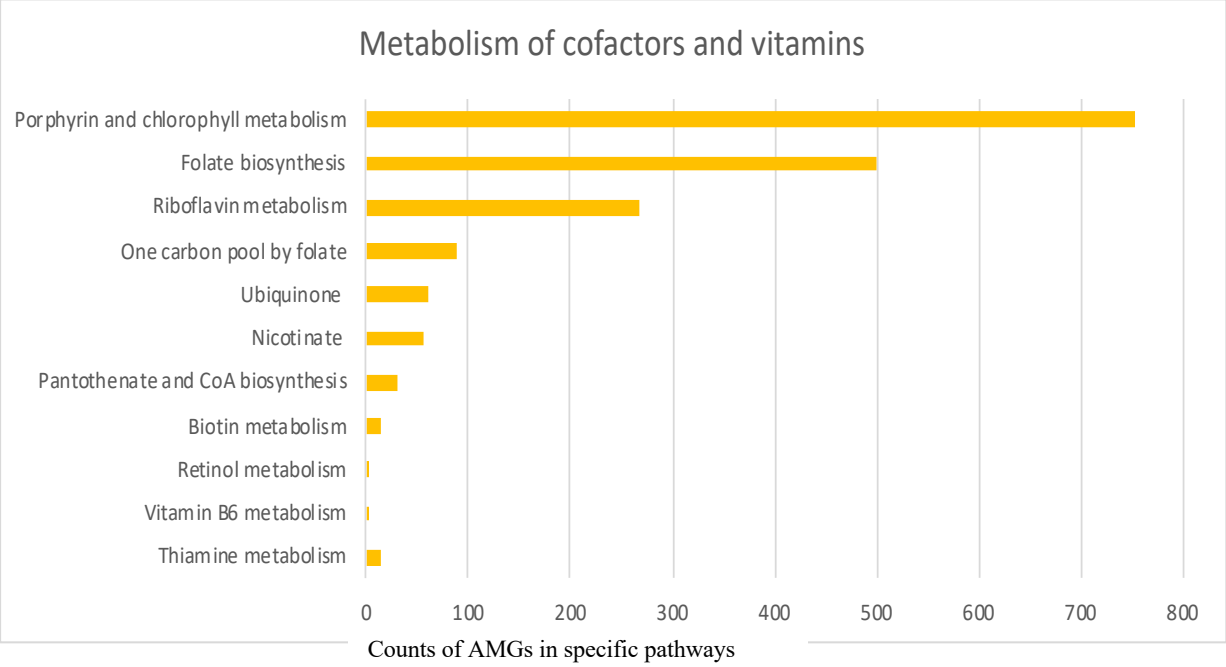


Figure 8. The total counts of AMGs involved in cofactor and vitamin metabolism. From these, the majority of these genes were those involved in chlorophyll and folate metabolism.

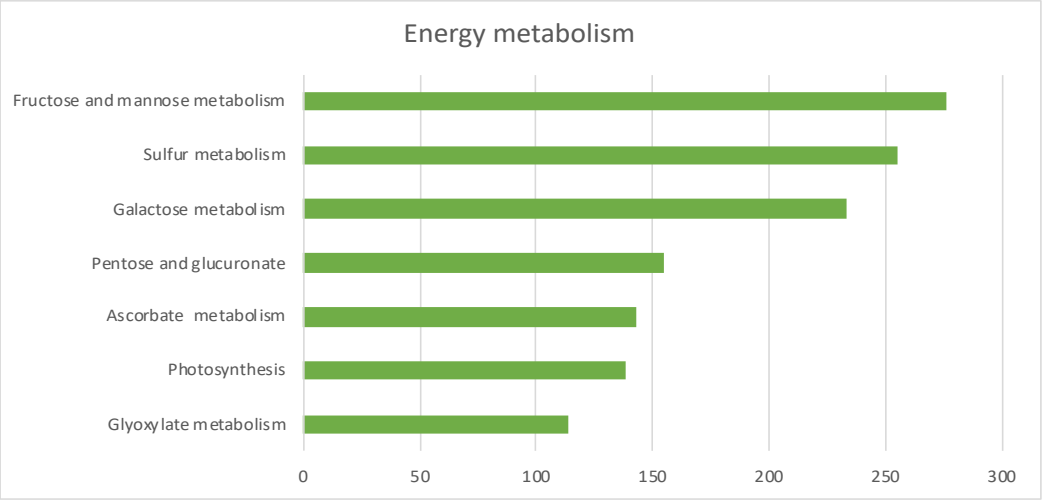


Figure 9. The total counts of AMGs that involved in energy metabolism. Viruses from this study appear not to use photosynthesis as a primary energy source. These viruses obtain energy from various sources including sulfur, methane and nitrogen metabolisms.

Distribution of sulfur metabolizing AMGs

Due to the high abundance of sulfur metabolising genes in our metagenomes, we investigated the distribution of sulfur-metabolizing AMGs across the different sampling sites (Figure 10). We found that the majority of these genes were enriched in viruses recovered from the Agulhas Current and the Agulhas Return Current (52%). Sulfur metabolising AMGs included *cysH (*94), *msm*A *(*60), *fcc*A (59) *cys*K (17), *cys*C (13*), sox*Y (8), *apr*B (3), *dsr*A (1) and *dsr*C (1). We also found high proportions of these genes at sites within close proximity to the Agulhas Current including the South Tropical Front (Figure 10, Supplementary Table 2). The *cys*H and *msm*A genes were found at all the sampling sites. Furthermore, of the sulfur metabolising genes, adenylylsulfate reductase genes (*apr*B) have not been previously reported in viruses.

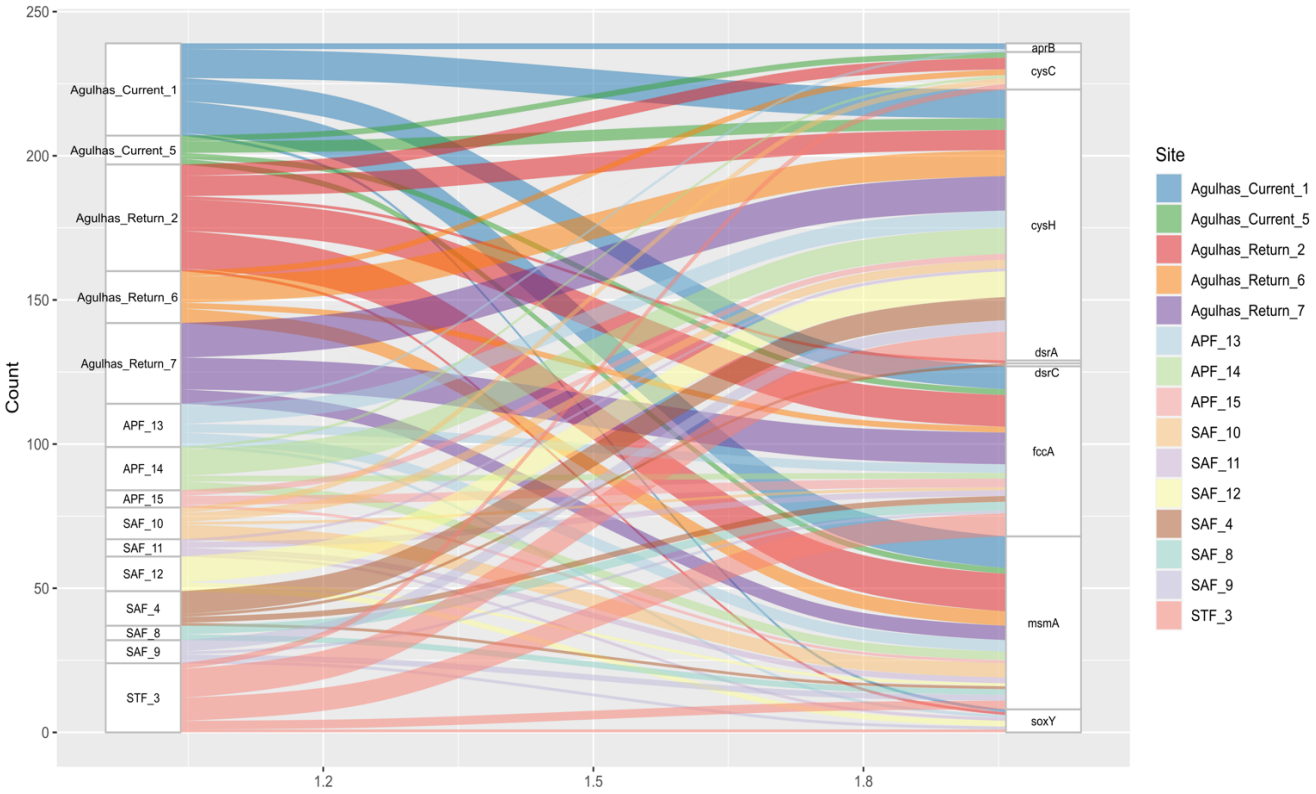
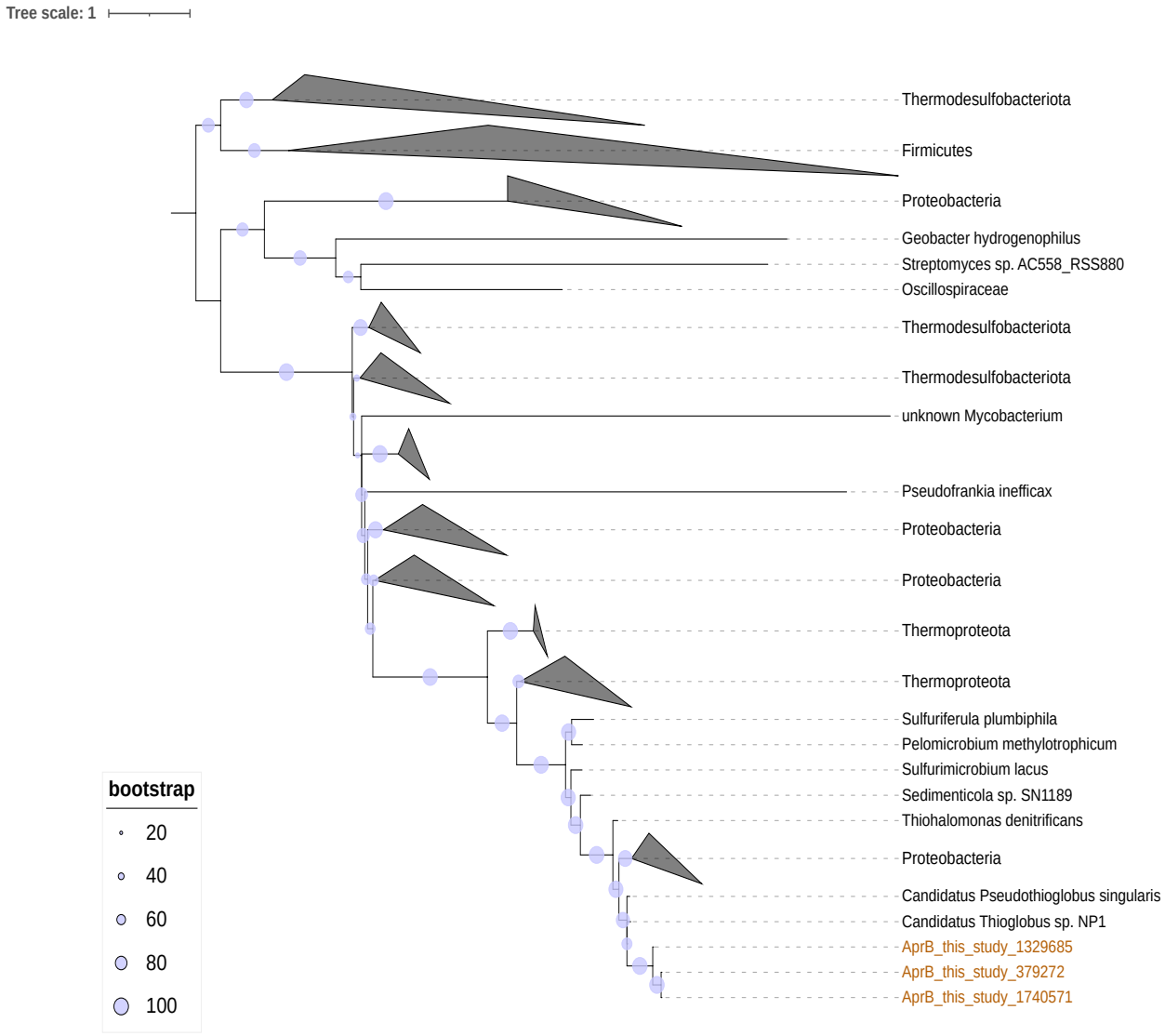


Figure 10. Alluvial plot showing the distribution of AMGs involved in sulfur metabolism, obtained from each sampling site. The majority of these genes were acquired from the Agulhas Regions. **AC**- Agulhas Current, **ARC**-Agulhas Return Current, **STF** -Sub-Tropical Front, SAF - Sub-Antarctic Front and **PF-**Polar Front.

Evolution and predicted structure of adenylylsulfate reductase (*aprB)* gene suggests acquisition from sulfur metabolizing bacteria.

A)



B)

Figure 11. A)-A maximum- likelihood tree constructed using AprB protein sequences from this study against AprB protein sequences from RefSeq. Viral AMGs found in this study were labelled in brown. **B)** Protein structure predictions of the aprB sequences found in this study. Confidence and coverage of two of the protein structures as

predicted by Phyre 2 was 99.8 and 98%, respectively. Confidence and coverage of the third aprB sequence was 99.1 and 68%. These scores suggests that the predicted protein structures are likely accurate.

Since the *apr*B gene has not been identified in viruses previously, we determined its evolution. For these analyses, we compared the amino acid sequences of *Apr*B from this study to those available in public repositories. These analysis revealed that sequences from this study clustered with AprB protein sequences, similar to those encoded by members of the *Thioglobus sp.,* which are known sulfur-oxidizing bacteria (Figure 11a). The results from Phyre 2 analysis suggest that the protein structure predictions may be a beta-barrel, which is similar to the crystal structure of adenylylsulfate reductase. The confidence score and coverage was 99.8% and 98%, respectively, for two of the three protein sequences (Figure 11b). This confirms that the predicted structure had high homology to the *Apr*B protein. Genomic maps of the contigs, containing the *apr*B gene, confirmed that these scaffolds were viral genomes as each contig contained viral sequences that play roles in either tail assembly, viral DNA binding or viral membrane fusion (Figure 12).

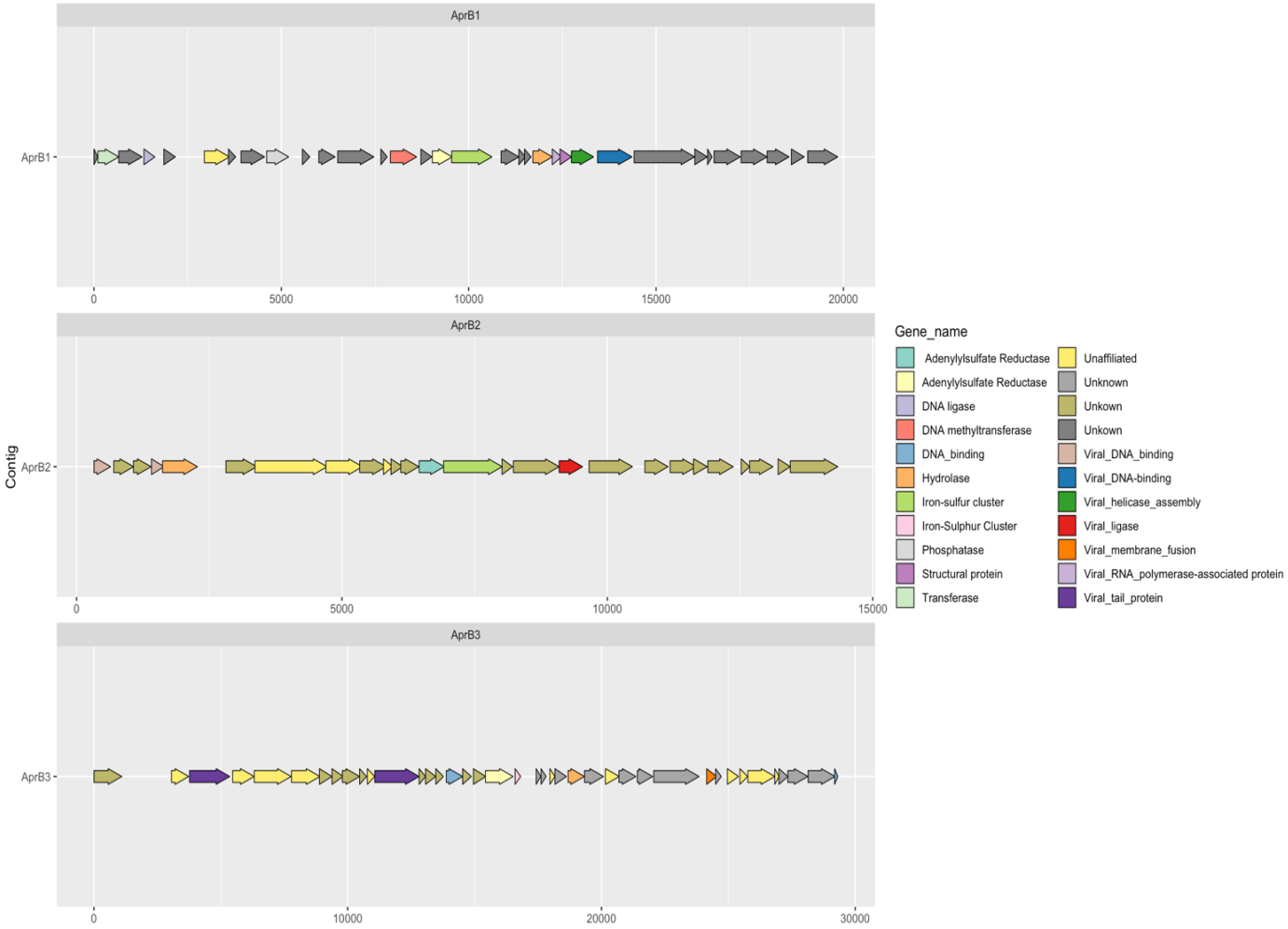


Figure 12. Genomic maps of contigs encoding *apr*B gene obtained from this study. The protein predictions were generated using Prodigal, and protein annotations were done with HHphred. Each contig contained viral genes which are involved in either viral tail assembly, viral DNA binding or viral membrane fusion.

Discussion

The discovery of AMGs has renewed interest in the role of viruses in global nutrient cycling and ecosystem function (5, 40). AMGs, encoded by viruses, alter the metabolism of their hosts, leading to significant contributions to biogeochemical recycling (1, 21, 41, 42). Despite the increasing evidence from studies from the Pacific and Atlantic Ocean, we lack comparative studies regarding the phylogeny and function of viral AMGs in some marine environments. This knowledge deficit is particularly true for understudied regions including the South Indian and Southern Ocean. Here, we provide data regarding the functional diversity and distribution of viral AMGs in the Agulhas regions and the Southern Ocean. We show that AMGs are remarkably

widespread and diverse in these marine environments. Our analysis further reveals that viruses in these regions encode a wide range of metabolic functions including photosynthesis, dissimilatory sulfur metabolism, carbon metabolism and vitamin/co-factor metabolism. This finding confirms that viruses have functional roles in these marine ecosystems and may substantially contribute to nutrient recycling. These dataset also establish a baseline and provide an overview of the diverse AMGs in the Agulhas Current and Southern Ocean.

The variation in the prevalence of AMGs between the Agulhas Current and the Southern Ocean may be linked to distinct environmental characteristics, such as temperature and nutrient availability (12, 13, 43). Warm tropical waters, such as those found in the Agulhas Current, have been shown to promote viral replication and viral -host interactions (44-46). Previous studies suggest that increased interactions may increase horizontal gene transfer and the acquisition of AMGs (2, 5). Moreover, nutrient upwelling from the high velocity winds in the Agulhas Current, increases primary production and microbial biomass (47). This may increase opportunities for viral infection, along with the expression of AMGs (48, 49). Thus, the high abundance of AMGs in the Agulhas Current and Agulhas Return Current suggests that these regions might be hotspots for biogeochemical processes influenced by viral activity. The results suggest that viruses may disproportionately contribute to nutrient recycling in this region.

Viral metabolism targets key rate limiting cellular processes during infection (50). AMGs are expressed in such a way that favours viral replication (8, 23). Indeed, the DNA methyltransferase 1 *(DNMT1/dcm)* gene, which encodes for a methyltransferase enzyme, was the most abundant AMG in this study (51). These enzymes are found in all prokaryotes and are often associated with restriction endonucleases, which help to protect bacterial cells from foreign DNA (13, 51). In bacteriophages, the *DNMT1* gene is not associated with restriction endonucleases, but is instead involved in regulatory activities that protect the phage DNA from being digested by the host (13, 52). This suggest that viruses in the Agulhas Current and Southern Ocean potentially use AMGs to evade host restriction mechanisms, which could increase viral fitness in these ecosystems (13).

Under the category of energy metabolism, we found more AMGs linked to sulfur metabolism than those implicated in photosynthesis. This finding is somewhat surprising as the samples were obtained from the euphotic zone, where many microbes rely on the energy generated from photosynthesis as their primary energy source (53). However, the rapid turnover and the four dimensional nature of these marine environments, may explain this discrepancy. Of the sulfur metabolising genes, phosphoadenosine phosphosulfate reductase (*cysH*) was the most abundant. This gene is involved in a subpathway that synthesises sulfite from sulfate, and is central to the sulfate reduction pathway (54, 55). *CysH* has been identified previously in pelagiphages that infect members of the SAR11 clade (56). This finding by S. Du et al. (56) was unexpected as members of the SAR11 clade were known to lack the *cys*H and other genes required for assimilatory sulfate reduction (57). Therefore, the presence of the *cys*H gene, in the pelagiphages, suggests a potential transfer from other bacteria or phages. More recently, the *cys*H gene has also been identified in viruses from both deep and hadal zones of the ocean, cold seep sediments and hypoxic waters (34, 37-39). In these regions, the *cys*H gene has been predicted to aid in the biosynthesis of amino acids and nitrogen fixation (39). The specific function of this gene during phage infection remains unclear. However, it is thought that bacteriophages divert sulfur from the bacterial energy metabolism towards amino acid synthesis for viral particle production, and that the *cys*H gene may be involved in this process (38). The presence of abundant *cys*H genes, in our viromes, suggests that viruses in these regions may be diverting sulfur from bacterial energy metabolism towards viral particle production, which may impact the biogeochemical cycling of sulfur (35).

Other sulfur oxidising genes found in our viral genomes, include of *dsr*C and dsrA. In sulfur metabolising bacteria, sulfide oxidation is a crucial step in the pathway that yields the most electrons (six electrons) for ATP production (35, 58). However, this step is rate-limited, by the co-activity of *Dsr*C and *Dsr*A, or the saturation of the *Dsr*AB enzyme complex (59, 60). During phage infection, the expression of phage-encoded *Dsr*C and/or *Dsr*A could supplement the rate-limiting step, potentially increasing the rate and ATP yield of the reaction (39). Furthermore, the presence of methanesulfonate monooxygenase subunit alpha (*msmA*), and cytochrome subunit of sulfide dehydrogenase (*fccA*) was consistent with previous work that identified these

genes in the epipelagic zine of the ocean (39). Both the *msm*A and *fcc*A genes encode enzymes which catalyse key steps in the sulfur oxidation pathway (61-63). The ability to enhance sulfide oxidation, and increase ATP production, may provide a competitive advantage for the viruses in the Agulhas Current system, potentially promoting viral replication and abundance in this region.

The positive identification of the adenylylsulfate reductase gene (*aprB*) in viral genomes is noteworthy. This is because this gene has not been previously reported in viruses. The *aprB* gene is involved in the sulfate dissimilatory reduction pathway, where inorganic sulfate is reduced to sulfide (64). The first step the pathway includes activation of a stable molecule of sulfate by reacting with ATP catalysed by sulfate adenylyltransferase (Sat) (65). From this, Adenosine 5'- phosphosulfate (APS) is generated and is reduced to sulfite by adenylylsulfate reductase (AprBA) (64, 66). AprBA is an αβ heterodimer containing two subunits, AprA and AprB (66). The final step in the pathway is the reduction of sulfite to sulfide by dissimilatory sulfite reductase (DsrABC) (67, 68). Phylogenetic analysis revealed that the *aprB* gene is closely related to AprB protein sequences from sulfur-oxidizing bacteria, such as *Thioglobus* sp. This suggests that this gene may have been horizontally acquired between viruses and sulfur-oxidising bacteria (2, 7). Furthermore, the presence of viral tail assembly, viral DNA binding, and viral membrane fusion genes in the contigs containing the *aprB* gene supports the idea that viruses may operate as vehicles for transmitting sulfur metabolism-related genes between organisms in the marine environment.

Based on the prevalence of genes involved in sulfur metabolism, viruses in the Agulhas current and Southern Oceans potentially contribute to the biogeochemical cycling of sulfur. To the best of our knowledge, this study is the first to report on the distribution and potential ecological significance of AMGs in these regions. Our findings align with recent studies on wetlands, hydrothermal environments, freshwater lakes, and other oceans, which demonstrated the significant contribution of viral AMGs to sulfur cycling (11, 34, 35, 37). The presence of viral *dsr*A*, dsrC, and soxY* genes supports the prediction that viral auxiliary metabolism potentially targets bottleneck steps in host metabolism to favour viral survival (29, 39). A potential utility of these sulfur AMGs in viruses may be to increase energy production, which

potentially increases the rates of dNTPs synthesis for viral replication (8). Therefore, bacteriophages in these regions may benefit more than the host from having an additional copy of the metabolic gene.

Conclusion

To conclude, we investigated the role of viral auxiliary metabolic genes in marine ecosystems. The study focused on the understudied and important sites within the vicinity of the Agulhas Current system and the Southern Oceans. The results confirm the significance of viral AMGs in shaping marine ecosystems. Furthermore, the study offers valuable insights regarding the functional diversity of AMGs in marine regions. Bacteriophages, which infect sulfur metabolising bacteria, may influence the biogeochemical cycling by redirecting energy from reduced sulfur pools to replication (38). Without viral infection, the energy generated by reduced sulfur pools would typically be used for primary production to fuel microbial cell growth. This energy would then be transferred higher up the food chain to grazers (35). Moreover, the identification of novel AMGs, such as *apr*B, expands our understanding of viral evolution and its potential implications on sulfur biogeochemical processes in marine ecosystems. To validate these results, methods like quantitative reverse transcription–PCR (qRT-PCR) may be used to quantify the expression of AMGs in these regions. Furthermore, proteomics may elucidate the precise mechanisms by which viral proteins modulate host cell physiology and ecosystem function in sulfur-metabolizing virocells. Taken together, the results provide a strong foundation for understanding virus-mediated nutrient cycling in climate critical environments. We emphasize the importance of incorporating viral contributions into ecosystem models, as viruses play critical roles in the biogeochemical cycling of elements.

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