Abundance and distribution of bacteria and viruses in the various ecotypes of the Hawaiian Islands

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Non-technical Abstract

Viruses are known to be the most abundant biological entity in ocean waters except for nano-viruses and virophages. As such, they play important roles in food webs and nutrient, mineral, and chemical cycling in the world's oceans. This project focuses on determining the variation in abundance of viruses and bacteria with depth in the waters around the Hawaiian Islands. The relationship between viruses and bacteria can be used to determine the effectiveness of transfers within food webs and other chemical cycles. One of the processes affected by viruses is known as the carbon cycle. This is an area of research that concentrates on the ways in which carbon is moved from the land to the atmosphere to the ocean to sediments at the bottom of the ocean. The cycle is important to global climate change studies because CO_2 is one of the main greenhouse gases warming up our atmosphere. Viruses are particularly interesting at the moment because their interaction with bacteria in the biological pump of the carbon cycle may be one way in which the rate of transfer of carbon from the atmosphere to ocean depths is increased. The hypotheses of this project were confirmed when a decrease in abundance of bacteria and viruses with depth was found. There was also a correlation between abundance of viral like particles and cells and the oxygen levels available. Nutrients were found to be inversely related to the presence of bacteria and viruses and not at all related to the ratio of the two entities. Levels of chlorophyll A, a proxy measurement for the number of phytoplankton in the water, were also measured, but were not correlated with bacterial or viral abundance. This was surprising because not all viral variation is explained by changes in bacterial abundance, and phytoplankton are another type of host for viruses. It is important to quantify all the relationships in these complex systems in order to better understand the processes which control our oceans, and therefore the world.

Abstract

Viruses and bacteria are highly abundant in the marine realm. Many studies have shown that viruses often exceed bacteria in abundance by an order of magnitude. This study utilized epi-fluorescence microscopy and DAPI and SYBR stain to quantify abundance of the two entities in the waters of the Hawaiian Islands. Cellular abundance ranged from 2.67×10^4 cells/mL to 3.87×10^5 cells/mL. Viral abundance ranged from 1.46×10^5 VLPs/mL to 1.74×10^6 VLPs/mL. Abundance of viral like particles and bacteria was found to have a correlation coefficient of 0.8916 indicating a strong positive relationship. The virus to bacteria ratio was calculated, but did not vary with depth, oxygen content or nutrient availability as had been anticipated. Accurate abundance estimates of viruses and bacteria throughout their environment are necessary for correct representations of the systems which drive global processes such as photosynthesis, respiration and carbon transport and sequestration.

Introduction

Numerically, viruses are the single most abundant biological entity in the marine ecosystem (Suttle, 2007). A conservative estimate counts ~ 3×10^6 viruses per mL in the deep sea and up to 10^8 viruses per mL in coastal waters (Suttle, 2005, See Appendix 1 for more estimates). If all these viruses were spread end to end, they would stretch 100 times the length of our galaxy (Suttle, 2005). While they remain microscopic, their sheer profusion allows them to play an important role in the globe's biogeochemical cycles.

Often viruses are studied according to their relation to the microbial loop and their impact on the efficiency of carbon sequestration. The microbial loop is a series of trophic relationships between the various groups of organisms that make up the marine food web (Azam et al., 1983). Figure 1 details how upwelling from deep water brings inorganic nutrients for phytoplankton to use. The phytoplankton are in turn consumed by organisms of higher trophic levels. As the phytoplankton decompose, either from predation or lack of sunlight and nutrients, compounds are recycled back into the pool of dissolved organic matter (DOM). This DOM can then be further decomposed by bacterioplankton and returned to an inorganic state. When there is a sudden influx of matter from the phytoplankton

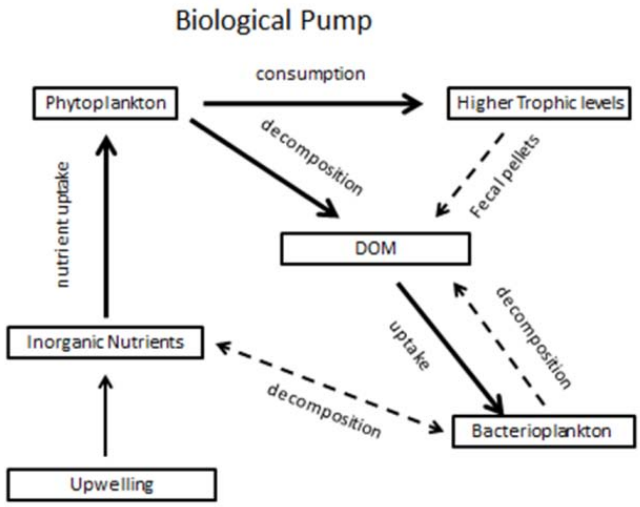


Figure 1. A schematic of the biological pump showing relationships between different components. DOM is dissolved organic matter, bold arrows are direct connections or predation while dashed arrows are indirect connections; the result of decomposition or sloppy feeding.

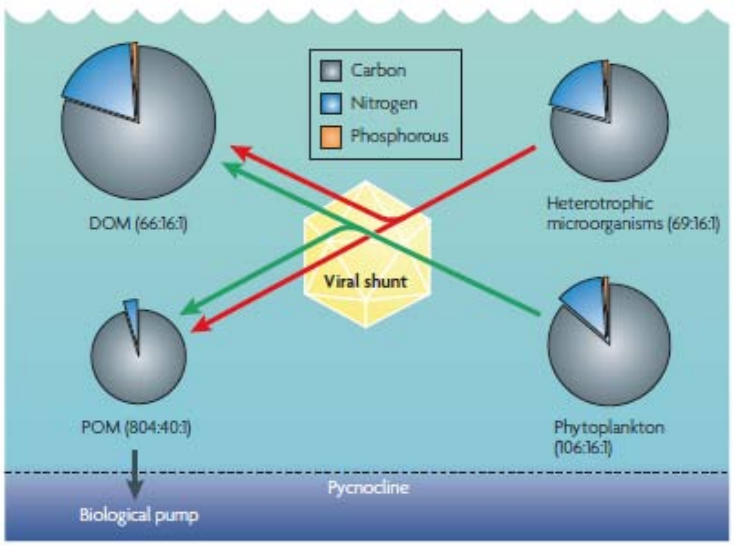


Figure 2. The red and green arrows represent the movement of material from heterotrophs and photoautotrophs (respectively) to the pools of particulate organic matter (POM) and dissolved organic matter (DOM) via the viral shunt. The chemical compositions of the POM and DOM pools are not necessarily the same as the organisms from which they are derived due to a stoichiometric effect associated with the transfer. Amino acids and nucleic acids are typically recycled in the photic zone while more carbon rich materials such as cell walls are exported to depth. Therefore, the matter sent to deep waters by the viral shunt is probably more carbon rich than the material from which it was derived. The biological pump increases in efficiency. The numbers in parentheses are the estimated ratios of carbon: nitrogen: phosphorus (in atoms). (reproduced from Suttle 2007)

and higher trophic levels, not everything can be recycled and some detritus is sequestered at depth. However, when the viral shunt is added to the system, sequestration of carbon at depth becomes a much more efficient process (Fig. 2, Suttle 2007). When viruses cause cell lysis, amino acids and nucleic acids released in the burst can be recycled into the DOM pool. The more carbon rich materials, such as cell walls, are exported to depth instead of passing through the bacterioplankton. Since less carbon is being recycled into the DOM, more must be drawn down from the atmosphere by the phytoplankton, hence increasing the efficiency of the biological pump. With as much as one-quarter of the organic carbon present in the world's oceans being recycled through the viral shunt, it is critically important to quantify the factors involved in viral lysis and incorporate these processes into models of global biogeochemical cycles (Wilhelm and Suttle, 1999).

Another role viruses play in manipulating the marine environment involves the "kill the winner" hypothesis. This theory articulates the idea that viral infection is the agent that prevents any single species of bacteria from dominating the whole community (Thingstad and Lignell, 1997). "Kill the winner" is particularly applicable during spring bloom cycles of phytoplankton

and bacteria. The surge in available hosts allows for an increase in free-living viruses and thus increased rates of infection. When this happens, so much cell lysis takes place that not everything can be remineralized, and a significant portion of material sinks to the benthos, thereby increasing efficiency of the microbial loop and adding weight to the claim of viral impact on the system. However, with this comes an implied dependency on the availability of host cells in the water column. This leads to the hypothesis that bacterial and viral abundance will be correlated and decrease with depth as chlorophyll a concentration, nutrient availability and host cell presence decreases. More explicitly, bacterial abundance will decrease due to lack of nutrients while viruses will adjust infection strategies to account for reduced availability of host cells (Hara et al. 1996). A correlation with oxygen is also expected because of the need for bacteria to respire, and the need for bacteria as viral hosts. Bacteria are not the only hosts available to viruses – in fact – they will infect everything from other viruses to blue whales (Suttle 2007). However, Cochlan et al. (1993) found that bacterial abundance alone could explain 67% of spatial variability in virus numbers. This suggestion of bacteria as the main host of viruses in the marine environment leads to the focus on bacterial abundance in this study. The R^2 value of the correlation between viruses and bacteria will be used to determine the dependence of viruses on bacterial hosts in the Hawaiian Islands. Accurate abundance estimates of bacteria and viruses

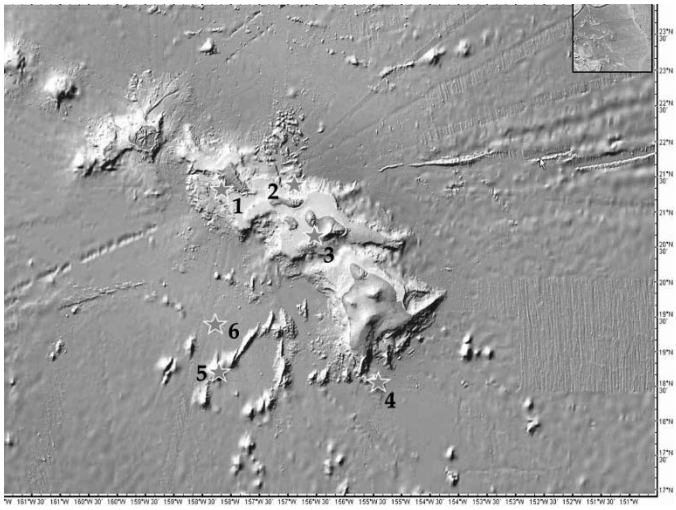


Figure 3. Station 1 Oahu (21 20.3940,158 11.2276) is 900m deep. Station 2, Molokai (21 19.0531,156 57.2617) is 1930m deep. Station 3, Molokini (20 35.55,156 28.68) is 240m deep. Station 4 is Loihi (18 54.4694,155 15.6195) is 1170m deep. Station 5 Cross Seamount (18 42.8722,158 15.9882) is 380m deep. Station 6 Open Ocean(18 56.2700,158 25.1740) is 4500m deep.

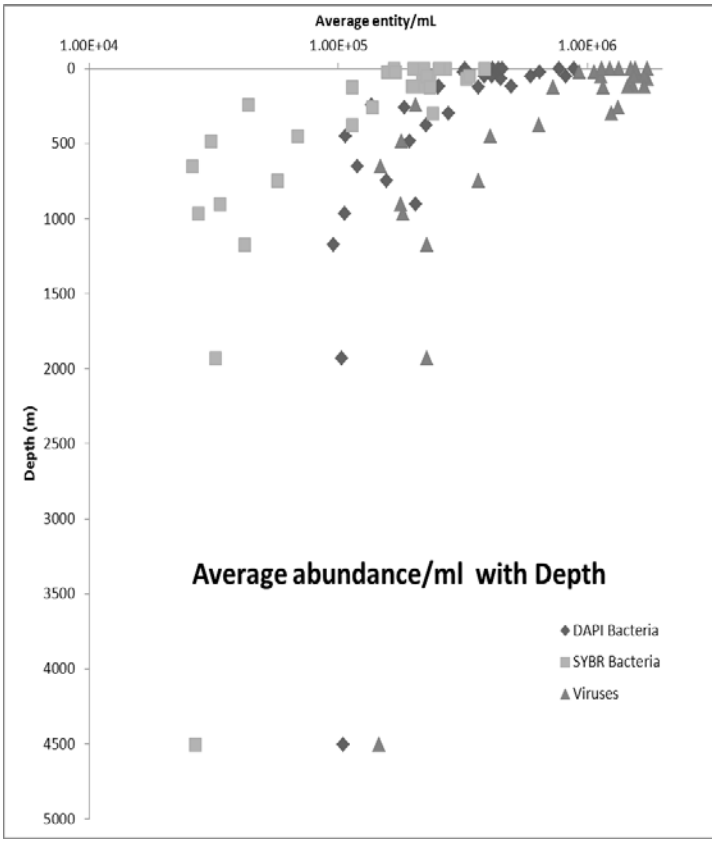
throughout their environment, including those exemplified by Hawaiian ecotypes, are necessary for correct representations of the systems which drive global processes such as photosynthesis, respiration and carbon transport and sequestration.

Methods

In order to accurately survey the various ecotypes around the Hawaiian Islands, several representative stations were chosen (See Fig. 3.). The first sample point was Pang 3, off the southeastern coast of Oahu, a high human-impact area and near-shore environment. Station 2 was Jim Core 4, off the northern coast of Molokai Island (hereafter referred to as Molokai). It is an area of interesting geological features, namely underwater canyons. It is also the only site north of the island chain. The third station was the Molokini sunken crater; a human impacted zone, but still uninhabited, also nearshore. Station 4 was Loihi seamount, a hydrothermally active zone. Station 5 was the Cross Seamount. Station 6 was an arbitrary location in the open ocean, taken as a control for variation in other sample points. All samples were taken aboard the R/V Thomas G. Thompson from December 27, 2010 through January 4, 2011.

The total abundance of bacteria and viruses in the water column were determined via DAPI and SYBR staining using epifluorescence microscopy. Water samples of 20ml were taken in scintillation vials at the surface, 5m from the seafloor, above, below, and within the chlorophyll maximum at each station. These were taken via niskin bottles on a rosette with CTD package. Samples were also taken for chlorophyll and nutrient analyses. Samples were fixed using 2.5 ml of paraformaldehyde to preserve bacteria and viruses. DAPI staining was started on board ship using a Hoeffer box as the filtration unit, remaining counts were completed in the Baross Laboratory at the University of Washington. For bacteria counts, 5mL of seawater was filtered through a 0.2μm black poly-carbonate filter. Approximately 15 drops of DAPI solution were

added to filter, and left to stand in the dark for 15 minutes (Porter and Feig, 1980). The DAPI was filtered through, and then the filter was mounted on a slide using paraffin oil. The slide was then examined via epifluorescence microscopy and cells within the grid were counted. Counts continued until 20 fields of view or 200 cells had been counted, whichever came first (Kirchmann et al., 1982). For SYBR counts, 1-5 ml of seawater were filtered through 0.02µm Anodisc filters. The filters were then removed from the Hoeffer box and placed in a petri dish over a 20µL drop of 1-5x SYBR Gold solution, covered, and left to stand in dark for 15 minutes (Tuma et al., 1999, Noble and Fuhrman, 1998). Next, the filters were wicked dry with a kim-wipe and mounted on a slide using 30µL of PBS glycerol. The same counting protocol was used. Results were averaged and regression and Pearson product-moment correlations were used to analyze the data for statistical significance.



Chlorophyll was collected at depths ranging from the surface to below the chlorophyll maximum at each station. At each depth, three 0.136L samples were taken and processed using the spectriphotometric method as outlined in Lorenzen 1967. The results were then averaged and used to calibrate the CTD fluorometer results.

Nutrient samples were collected in triplicate at each depth and sent to the University of Washington's Marine Chemistry laboratory for processing.

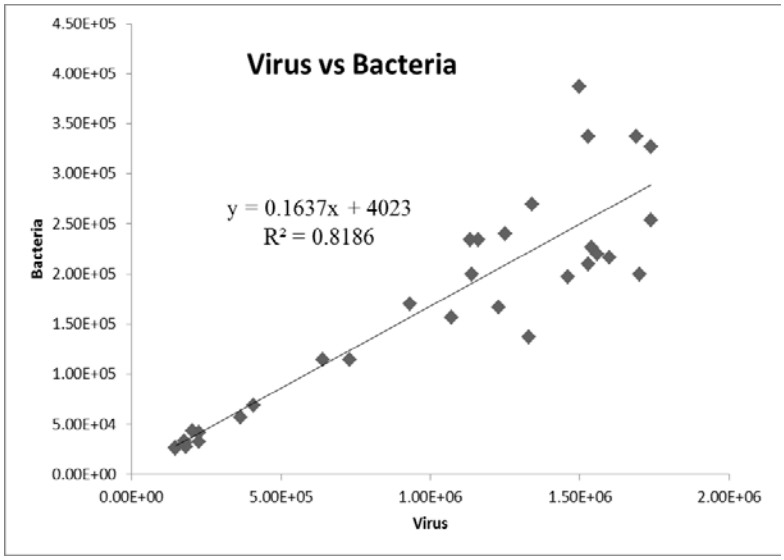


Figure 4. The average abundance plotted against depth shows the overall trend expected; a decrease in abundance with depth for all entities, and a higher abundance of virus-like particles than cell-like particles

Results

The general trend of the cell counts has been a decrease of abundance with depth (Fig.4). There is a discrepancy in the number of bacterial like cells observed between the two counting methods. In order to minimize possible sources of error, only the SYBR gold counts, in which cells and viruses were counted simultaneously, will be regarded as significant. The DAPI counts serve only to re-iterate the general decreasing trend in abundance.

Figure 5. Plotted against each other, viruses and bacteria (cell/ml) exhibit a linear relationship in abundance

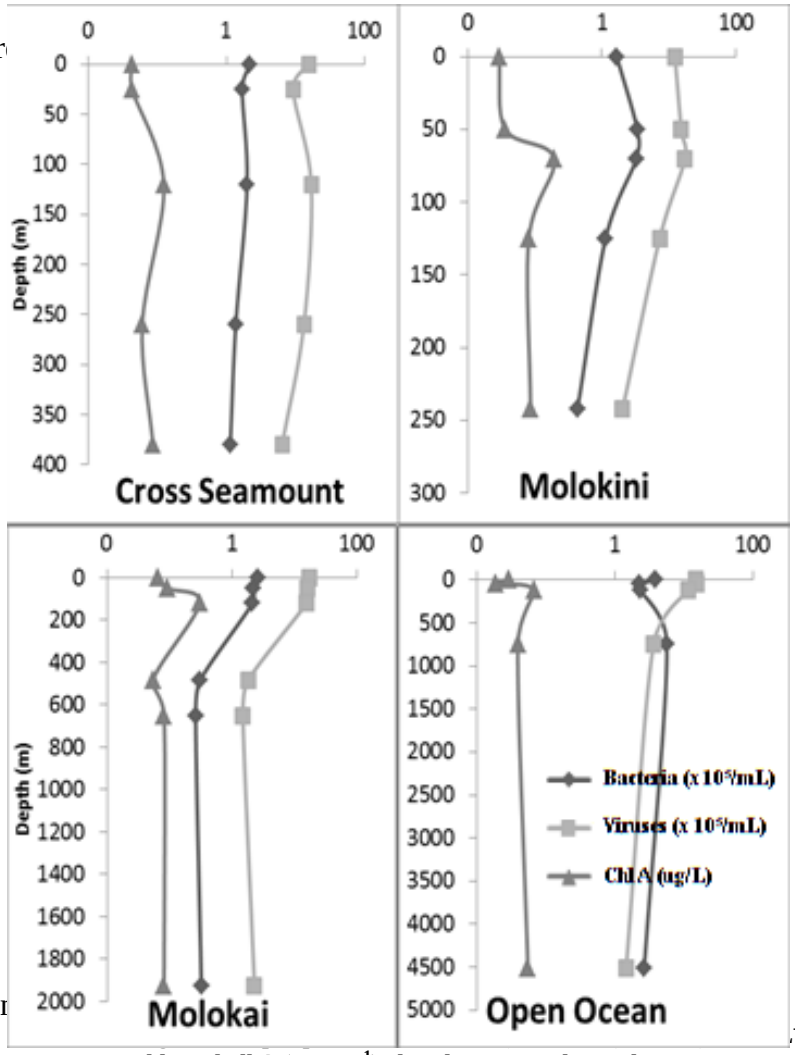
The cellular abundance ranged from a low of 1.67×10^5 cells/mL at Molokini to a high of 3.87×10^5 cells/mL at the open ocean control in surface waters, and from a low of 2.67×10^4 cells/mL at the control to a high of 1.14×10^5 cells/mL at Cross Seamount at 5m from the bottom. Viral like particulate (VLP) abundance ranged from a high of 1.74×10^6 VLPs/mL at Molokai to a low of 1.14×10^6 VLPs/mL at Loihi in surface waters and a high of 6.41×10^5 VLPs/mL at Cross to a low of 1.46×10^5 VLPs/mL at the open ocean control at depth.

There was little to no observed variation in the virus to bacteria ratio (VBR) with depth. The ratio itself had an R^2 value of 0.8186 indicating a strong relationship between viral and bacterial abundance. A strong positive correlation of 0.905 was found between VLPs and cells. However, when the VBR is plotted versus depth, the R^2 value is 0.0033 indicating that only 3.3% of the observed decline can be attributed to changes in depth.

Table 1. The Pearson product-moment correlation coefficient showing the linear dependence between variables. P-values of <0.02 are significant, p-values of >0.10 are not significant. D.f. = 30

	Virus	P-value	Bacteria	P-value	VBR	P-value
O2	0.839	<.01	0.762	<.01	0.172	>.10
[PO4]	-0.840	<.01	-0.768	<.01	-0.136	>.10
[Si(OH)4]	-0.785	<.01	-0.712	<.01	-0.120	>.10
[NO3]	-0.846	<.01	-0.769	<.01	-0.150	>.10
[NO2]	-0.462	<.01	-0.441	<.02	-0.026	>.10
[NH4]	-0.041	>.10	-0.112	>.10	0.078	>.10
Chl A	0.215	>.10	0.077	>.10	0.244	>.10

The Pearson product-moment correlation coefficient was used to measure the strength of linear dependence between two variables (Table 1). A coefficient of +1 indicates a positive correlation. A coefficient of 0 indicates no correlation while a -1 indicates an inverse relationship. On their own, viral and bacterial abundances averaged over all stations are both significantly correlated with oxygen levels, but inversely related to all nutrients besides ammonia with which they have no r any of the measured variables. There is also no overall significant relationship detected between bacteria, viruses and chlorophyll a concentration s. However, there was sor variation in correlations between stations (Fig. 6, Appendix 2). Bacterial and viral abundance are closely correlated at all stations, and while chlorophyll A concentrations visually track this relationship, Molokini is the only station with a significant correlation (R=0.380 for bacteria, R=0.406 for viruses, P-value <0.05).



chlorophyll A (µg mL^{-1}) abundances on a log scale.

The open ocean control is the only station where bacterial abundance surpassed viral abundance at depth. The change in dominance occurred around the depth of 750m. Nutrients also varied by station, see appendix 2 for details.

Discussion

In the past, studies have been able to show a relationship between viral and bacterial communities in the water column (Culley and Welschmeyer, 2002, Hara, S. et al. 1996). Culley and Welshmeyer (2002) found that virus-like particles and prokaryote-like particles were more abundant in neritic regions as opposed to the open ocean, and that overall abundance was highest in surface waters and decreased with depth. This study found comparable results, i.e. a decrease in bacterial and viral abundance with depth. Total cell counts were also in the near-island environments of Molokini and Molokai as compared to Cross Seamount and the Open ocean control. This agrees with Cochlan et al. (1993) who reported higher concentration of viruses in coastal environments. Interestingly, bacterial and viral abundance were more highly correlated with nutrients such as phosphate and nitrate in the near shore areas (R ~ .7-.9XX) of Molokai and Molokini than the open ocean control where R ~ .5-.6XX (Appendix 2). This implies a greater dependence in coastal waters on limiting nutrients and argues for high levels of competition due to higher species diversity. Limiting nutrients become more important in determining abundances when a larger community (in both numbers and diversity) are using the resources. A future study might look into the effects of upwelling, competition, a potentially greater range of host species and general higher productivity as causes of larger numbers of viruses in coastal waters.

Both bacteria and viruses were positively correlated with oxygen concentrations, a logical result as bacteria require oxygen for respiration. Therefore, with increased oxygen, an increased

abundance in bacteria allows for a larger number of viruses. Oxygen is also produced by diatoms and other phytoplankton so increased oxygen levels might indicate greater host diversity available for viruses.

The virus to bacteria ratio was expected to decrease with depth because of the life histories of the two types of organisms. In surface waters, the availability of viral hosts is usually so great that viruses can afford to be free-living (the lytic stage) for a time before infecting a cell. Food sources decrease with depth, resulting in lower bacterial abundance, thereby reducing the number of possible hosts for viruses. Viruses adjust their life cycle by incorporating themselves as prophages into host genomes where they can remain indefinitely through many replication cycles until conditions become favorable to resume the lytic cycle (Rohwer and Thurber, 2009). Therefore, a decrease in the VBR is not proof of fewer viruses present per bacteria, but rather an indication of a life-strategy change. The only station where this relationship was observed was at the open ocean control. At every other station, the virus to bacteria ratio did not decrease in deeper waters as expected (Fig. 6). Instead, the ratio hovered between 4-7 VLPs present per bacteria regardless of depth. Other studies have found relatively similar numbers, but have not quantified presence or absence of support for changes in viral lifestyle. A study of the oligotrophic Alboran Sea (Alonso et al, 2001) found VBRs of 1.4-20.0 at the sub-surface chlorophyll maximum. Hara et al. (1996) compared subarctic to subtropical waters found a range of 1.1 to 7.4 in the subarctic VBR and 1.0 to 8.7 in the subtropics throughout the entire water column. Since only the open ocean station showed the reversal in virus and bacterial dominance, it is possible that there have been too many assumptions made about the importance of lysogeny. A potential influence on life cycle stage might be the greater availability of nutrients in the near-shore and near-seamount stations compared to the open ocean. Perhaps the relationship

described by Rohwer and Thurber (2009) is more prevalent in sediments as opposed to open water, or conditions at the other stations were not stressful enough for viruses to transition from the lytic to the prophage stage.

Viral abundance is affected by rates of viral production and loss, which in turn can be influenced by burst size, frequency of cell infection, diversity of hosts, UV radiation, and viral decay rates (Clasen et al., 2008). The R^2 value of the correlation between viruses and bacteria reveals the percent of variation in viral abundance that can be attributed to the variation in bacterial numbers (Table 2, Cochlan, 1993). Averaged over all stations, 81.9% of all viral variation can be explained by the presence of bacteria. At 61%, Cross Seamount had the least amount of its variation explained by bacteria, while Molokai at 99%, had the most amount of variation accounted for. While the R^2 value at Cross is only slightly less than the 67% reported in Cochlan et al. (1993), it is still surprisingly low compared to the other stations. Seamounts are interesting ecosystems because they experience the upwelling of nutrients from depth while remaining isolated at sea and not having as many land-based inputs as a coastal environment. This leads to differences in the biological communities of the three systems (coastal, open ocean, and seamount) which may affect the types of hosts available for viruses. A possible explanation for the discrepancy in R^2 values could be the presence of viruses specialized to infect zooplankton and other macroscopic organisms at Cross seamount. This specialization would help account for less abundant bacterial hosts (as compared to open ocean and coastal environments).

Table 2. R^2 value of the correlation between bacterial and viral abundance by station. P-value <0.01

Station	R^2
Cross	0.616
Molokai	0.996
Loihi	0.930
Molokini	0.900
Open Ocean	0.830
Pang3	0.926
All	0.819

Cross, Molokai, Loihi and Pang3 had no significant correlation with chlorophyll A. However, since at most, there is 29% of variation left to be potentially attributed to Chl A, low correlation values are not surprising (Table 1, Appendix 1). A positive correlation between viruses and the major host organisms may be expected, since a higher density of host cells increases the likelihood of infection and proliferation. Had an inverse correlation been found, this might have been evidence for predator-prey cycling. However, this is unlikely given the complexity of a community with numerous hosts available (Cochlan, 1993).

Conclusions

To summarize, the hypothesis of bacterial and viral abundance decreasing with depth was supported. However, the VBR decreasing with depth only occurred at the open ocean station and the VBR was only correlated with depth at Molokini. Total abundance of VLPs and bacteria were strongly correlated with each other and oxygen availability and inversely related to the presence of nutrients. The VBR was not significantly influenced by any of the parameters measured in this project. Overall, 81% of the total viral variation could be explained by the presence of bacterial hosts.

In conclusion, viruses are highly abundant in the waters around Hawaii, and as such, are an important force to keep in mind. In this study, the importance of bacterial hosts varied by as much as 30%, and that within a relatively small survey area. Primary hosts change from system to system and can be instrumental in defining the biogeochemical cycles of the area. In Hawaii, the main viral hosts were bacteria, key players in the biological pump and other marine cycles (Azam et al., 1983). It is crucial that the relationships in these complex systems be quantified in order to better understand the processes which control our oceans, and therefore the world.

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Appendices

Appendix 1. Distribution and abundance of marine viruses (reproduced from Wilhelm and Suttle, 1999)

Location	Viral abundance (virus particles/L)	Reference
Chesapeake Bay	$2.6–14 \times 10^9$	Wommack et al. 1992
Norwegian coast	$4–9 \times 10^{10}$	Bratbak et al. 1996
Japanese bays	$1.2–35 \times 10^9$	Hara et al. 1991
Western Gulf of Mexico		
Offshore	$3–4 \times 10^8$	Weinbauer and Suttle 1997
Coastal	$1.5–28.3 \times 10^{10}$	Weinbauer and Suttle 1997
Bermuda	$4.2–5 \times 10^8$	Jiang and Paul 1996
Florida coast	$2.7–11.5 \times 10^9$	Jiang and Paul 1996
Hawaiian Islands	$7.4–12.4 \times 10^8$	Jiang and Paul 1996
Santa Monica Bay	1×10^{10}	Noble and Fuhrman 1997
Long Island Sound	1×10^{11}	Proctor and Fuhrman 1990
Caribbean Sea	$1.9–4.8 \times 10^9$	Proctor and Fuhrman 1990
Bering and Chukchi Seas	$2.5–35 \times 10^9$	Steward et al. 1996

Appendix 2. Pearson product-moment correlation coefficients and relevant p-values

Bacteria																
	Depth	P-value	Oxygen	P-value	[PO4]	P-value	[Si(OH)4]	P-value	[NO3]	P-value	[NO2]	P-value	[NH4]	P-value	CHLA	P-value
Cross	-0.884	<.01	0.790	<.01	-0.840	<.01	-0.806	<.01	-0.817	<.01	-0.635	<.01	0.456	<.01	-0.054	>.10
Molokai	-0.717	<.01	0.950	<.01	-0.976	<.01	-0.824	<.01	-0.967	<.01	-0.944	<.01	-0.149	>.10	0.380	<.05
Loihi	-0.838	<.01	0.871	<.01	-0.878	<.01	-0.815	<.01	-0.875	<.01	-0.772	<.01	-0.155	>.10	-0.004	>.10
Molokini	-0.660	<.01	0.667	<.01	-0.717	<.01	-0.744	<.01	-0.743	<.01	-0.009	>.10	0.600	<.01	0.261	>.10
Open Ocea	-0.710	<.01	0.678	<.01	-0.599	<.01	-0.594	<.01	-0.603	<.01	-0.232	>.10	0.718	<.01	-0.357	<.05
Pang 3	-0.832	<.01	-	-	-0.823	<.01	-0.887	<.01	-0.845	<.01	-0.389	<.05	-0.448	<.02	0.196	>.10

Virus																
	Depth	P-value	Oxygen	P-value	[PO4]	P-value	[Si(OH)4]	P-value	[NO3]	P-value	[NO2]	P-value	[NH4]	P-value	CHLA	P-value
Cross	-0.532	<.01	0.736	<.01	-0.723	<.01	-0.753	<.01	-0.740	<.01	-0.265	>.10	0.185	>.10	0.229	>.10
Molokai	-0.707	<.01	0.957	<.01	-0.981	<.01	-0.820	<.01	-0.971	<.01	-0.941	<.01	-0.153	>.10	0.406	<.05
Loihi	-0.881	<.01	0.948	<.01	-0.953	<.01	-0.891	<.01	-0.951	<.01	-0.668	<.01	0.133	>.10	0.292	>.10
Molokini	-0.827	<.01	0.797	<.01	-0.845	<.01	-0.865	<.01	-0.879	<.01	-0.019	>.10	0.500	<.01	0.249	>.10
Open Ocea	-0.789	<.01	0.705	<.01	-0.683	<.01	-0.678	<.01	-0.684	<.01	-0.412	<.02	0.438	<.02	-0.486	<.01
Pang 3	-0.912	<.01	-	-	-0.902	<.01	-0.976	<.01	-0.924	<.01	-0.480	<.01	-0.572	<.01	0.082	>.10

VBR																
	Depth	P-value	Oxygen	P-value	[PO4]	P-value	[Si(OH)4]	P-value	[NO3]	P-value	[NO2]	P-value	[NH4]	P-value	CHLA	P-value
Cross	0.087	>.10	0.366	<.05	-0.272	>.10	-0.369	<.05	-0.331	<.10	0.173	>.10	-0.220	>.10	0.274	>.10
Molokai	-0.076	>.10	0.805	<.01	-0.685	<.01	-0.279	>.10	-0.652	<.01	-0.519	<.01	-0.402	<.05	0.464	<.01
Loihi	-0.021	>.10	0.237	>.10	-0.246	>.10	-0.301	<.10	-0.260	>.10	0.335	<.10	0.936	<.01	0.950	<.01
Molokini	-0.502	<.01	0.450	<.01	0.286	>.10	0.321	<.10	0.304	<.10	-0.130	>.10	-0.683	<.01	-0.259	>.10
Open Ocea	0.059	>.10	-0.448	<.02	-0.034	>.10	-0.031	>.10	-0.024	>.10	-0.474	<.01	-0.962	<.01	-0.279	>.10
Pang 3	-0.127	>.10	-		-0.126	>.10	-0.122	>.10	-0.116	>.10	-0.284	>.10	-0.300	<.10	-0.432	<.02