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Online program 'VIPCAL' for calculating lytic viral production and lysogenic cells based on a viral reduction approach

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Summary

Assessing viral production (VP) requires robust methodological settings combined with precise mathematical calculations. This contribution improves and standardizes mathematical calculations of VP and the assessment of the proportion of lysogenic cells in a sample. We present an online tool 'Viral Production Calculator' (VIPCAL, http://www.univie.ac.at/nuhag-php/vipcal) that calculates lytic production and the percentage of lysogenic cells based on data obtained from a viral reduction approach (VRA). The main advantage of our method lies in its universal applicability, even to different piecewise-linear curves. We demonstrate the application of our tool for calculating lytic VP and the proportion of lysogenic bacteria in an environmental sample. The program can also be used to calculate different parameters for estimating virus-induced mortality, including the percentage of lytically infected cells, lysis rate of bacteria, percentage of bacterial production lysed, proportion of bacterial loss per day, viral turnover time as well as dissolved organic carbon and nitrogen release. VIPCAL helps avoid differences in the calculation of VP and diverse viral parameters between studies and laboratories, which facilities interpretation of results. This tool represents a methodological step forward that can help improve our understanding of the role of viral activity in aquatic systems.

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Introduction

Aquatic viruses infect all members of the microbial food web and are significant biological agents in microbial processes (Fuhrman, 1999). Estimates of bacterial mortality due to phage production suggest that viruses can be responsible for up to 100% of bacterial mortality (Proctor and Fuhrman, 1990; Proctor *et al.*, 1993; Hennes and Simon, 1995; Noble and Fuhrman, 2000). Moreover, viruses may significantly impact natural bacterial abundance, productivity and community composition (Fuhrman and Schwalbach, 2003; Schwalbach *et al.*, 2004; Weinbauer, 2004; Hewson and Fuhrman, 2006; Bouvier and del Giorgio, 2007). Therefore, information on viral survival mechanisms and viral life strategies is of considerable interest. Viruses display different types of life cycles, the most common being lytic and lysogenic infections. In most aquatic environments, the lytic cycle is the dominant method of viral replication and ultimately destroys the infected cells. In the lysogenic cycle, the phage infects a host cell and the phage's genome typically remains in the host in a dormant stage known as a prophage. The prophage replicates along with the host until environmental stimuli such as UV radiation, temperature etc. cause proliferation of new phages via the lytic cycle (see review of Weinbauer, 2004). Lysogeny may be an important survival mechanism for viruses where host densities or resources are low (Wilson and Mann, 1997) or when the destruction rate of free phages is too high to allow for lytic replication (Lenski, 1988). Temperate viruses may affect host assemblage composition, either by lysis or by other mechanisms, e.g. by resistance to lytic virus infection (Hewson and Fuhrman, 2007a). Lysogeny also occurs in natural populations of the Cyanobacteria such as *Synechococcus* spp. (Ortmann *et al.*, 2002), and this interaction exhibits a seasonal pattern (McDaniel *et al.*, 2002).

Estimating the significance of the lysogenic pathway in natural viral populations requires determining the percentage of cells that are lysogens. Different approaches have been used to assess the frequency of lysogenic

cells: Jiang and Paul (1994) searched for lysogenic strains within cultures of marine bacteria using mitomycin C. Water samples were directly exposed to an inducing agent (sunlight, mitomycin C) to estimate the lysogenic frequency within bacteria (Jiang and Paul, 1996; Cochran and Paul, 1998; Williamson *et al.*, 2002). In these studies, lysogenic induction from natural bacterioplankton was investigated based on significant changes in viral (increase) and bacterial (decrease) abundances.

A very comprehensive overview of assumptions, advantages and disadvantages of different viral production (VP) techniques is given in Winget and colleagues (2005). The viral reduction approach (VRA) provides rapid and reproducible estimates of VP, which can be measured directly (Weinbauer and Suttle, 1996; McDaniel *et al.*, 2002; Weinbauer *et al.*, 2002; Wilhelm *et al.*, 2002; Hewson and Fuhrman, 2003; 2007b; Mei and Danovaro, 2004; Winter *et al.*, 2004; Helton *et al.*, 2005; Winget *et al.*, 2005; Williamson *et al.*, 2008). It allows direct observation of VP from natural microbial communities if certain assumptions are made (see Winget *et al.*, 2005). In this approach, the viral abundance of a water sample is reduced with virus-free water in order to minimize or even to stop new infections. This technique allows monitoring even small changes in the lytically produced viral abundance based on changes in viral direct counts (VDC) over time. Additionally, when estimating the amount of lysogens within bacteria, water samples have to be treated with an inducing agent. To induce the lytic cycle in lysogenized bacteria, mitomycin C and UV C radiation are the most powerful agents (Jiang and Paul, 1994; 1996; Wilcox and Fuhrman, 1994; Weinbauer and Suttle, 1996; Tapper and Hicks, 1998). If the inducing agent stimulates virus release, the abundance of viruses increases in the water samples.

In order to obtain a standardized, improved calculation, we programmed an online tool for estimating lytically and lysogenically produced viruses during a VRA. We demonstrate the application of our program for assessing lytic VP and the proportion of lysogenic cells in environmental samples. Furthermore, other viral parameters can be calculated, including the percentage of lytically infected cells, lysis rate of bacteria, percentage of bacterial production lysed, proportion of bacterial loss per day, viral turnover time as well as dissolved organic carbon and nitrogen release.

Results and discussion

Computations of VP based on a VRA

Lytic VP estimated by the VRA was initially published by Wilhelm and colleagues (2002). Viral production rates were determined from first-order regressions of viral abundance versus time, thus implying a continuous increase in viral abundance. Weinbauer and colleagues (2002) published a model for estimating lytic and lysogenic VP as follows: lytic VP is the difference between viral abundance in the stationary phase of the incubations without added mitomycin C and viral abundance at the start of the experiment. Lysogenic VP is the difference between viral abundance in the incubations with and without mitomycin C treatment. For lytic VP, Winter and colleagues (2004) developed the model further and discussed the potential occurrence of two peaks in viral abundance during the incubations. Lytic VP was calculated as the slope between the minimum (V_{min}) and the maximum viral abundance (V_{max}). For experiments in which two peaks of viral abundance occurred, VP was calculated according to the following formula:

$$VP = [(V_{max\,1} - V_{min\,1}) + (V_{max\,2} - V_{min\,2})]/(t_{max\,2} - t_{min\,1}),$$

where t = time (incubated hours). Lysogenic VP was not considered in this study.

Figure 1 displays different outcomes of VRAs throughout our experimental incubations. Comparing VDC (with and without mitomycin C) at the commencement of an experiment sometimes revealed a difference in these two treatments (Fig. 1B and C). Continuous growth in viral abundance, as observed by Wilhelm and colleagues (2002), was rarely found (Fig. 1A). In some of our incubations, viral abundance decreased or remained constant before it increased (Fig. 1B and C). Instead of a continuous increase, two or sometimes even three increases in viral abundance, followed by decreases, were observed (Fig. 1C). This was also reported by Winter and colleagues (2004) and Winget and colleagues (2005). Explanations for this viral response in experimental incubations include the release of viruses with different latent periods as well as new infection and cell death from viruses released in early lysis events.

Hence, our approach for the computation of the lytic VP rates in a VRA is as follows: let $VDC_{min\,1}$, $VDC_{max\,1}$, . . . , $VDC_{min\,n}$, $VDC_{max\,n}$ be the starting and end points and $t_{min\,1}$, $t_{max\,1}$, . . . , $t_{min\,n}$, $t_{max\,n}$ the time intervals between minimum and maximum values. Then,

$$VP = [(VDC_{max\,1} - VDC_{min\,1})/(t_{max\,1} - t_{min\,1}) + \ldots + (VDC_{max\,n} - VDC_{min\,n})/(t_{max\,n} - t_{min\,n})]/n$$

is the mean lytic VP for the experiment.

Regarding the viral abundances without mitomycin C, lytic VP in the experiment is determined for each time period with a net increase. Calculating the arithmetic mean of these VP rates gives the mean lytic VP per hour.

Using the above-mentioned models by Weinbauer and colleagues (2002) and Winter and colleagues (2004), problems in the computation of lysogenic VP can arise due to (i) different viral abundances at the onset of the

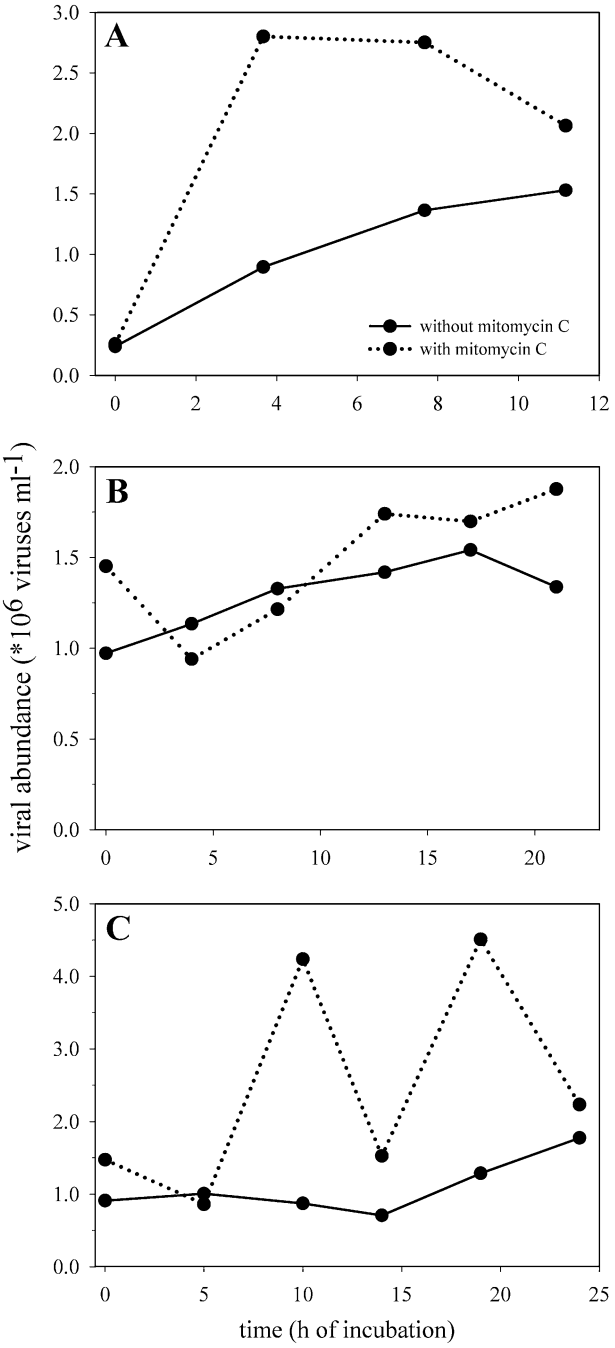


Fig. 1. Changes of viral abundances over time in three independent virus reduction experiments. To measure viral production, a virus reduction approach (VRA) was performed based on Weinbauer and colleagues (2002) and Wilhelm and colleagues (2002). Viruses and bacteria were stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) using a modified method of Noble and Fuhrman (1998). The study site and the sampling stations are published elsewhere (Peduzzi and Luef, 2008). Samples were taken from the Danube River on 23 May 2006 (A) and from an isolated subsystem known as Lobau on 29 May 2006 (B) and on 17 October 2005 (C). The outcome of the VRAs exhibits different shapes of curves.

experiment, (ii) the occurrence of two or more peaks in viral abundance, and (iii) different slopes of the two curves (with and without mitomycin C), sometimes causing several points of intersection.

Therefore, in our study, the lysogenic VP is represented by a difference curve, which is developed by calculating

$$VDC_{\text{mitomycin C}} - VDC_{\text{without mitomycin C}}$$

for the whole experiment.

Hence, the lysogenic VP rate for each net increase is computed from the difference curve as follows: let $VDC_{min\,1}$, $VDC_{max\,1}$, . . ., $VDC_{min\,n}$, $VDC_{max\,n}$ be the starting and end points and $t_{min\,1}$, $t_{max\,1}$, . . ., $t_{min\,n}$, $t_{max\,n}$ the time intervals between minimum and maximum values of the difference curve. Then,

$$VP = [(VDC_{max\,1} - VDC_{min\,1})/(t_{max\,1} - t_{min\,1}) + \ldots + (VDC_{max\,n} - VDC_{min\,n})/(t_{max\,n} - t_{min\,n})]$$

is the whole lysogenic VP within the experiment.

If the VDCs are higher in the treatment without mitomycin C than in the treatment with mitomycin C (which may occasionally occur), then the difference curve reaches values less than or equal to zero. In this case, the lysogenic VP of the experiment is simply computed from the time periods in which the difference curve has values greater than or equal to zero (Fig. 2C). Combining the approaches of Weinbauer and colleagues (2002) and Winter and colleagues (2004) sometimes yields negative values because the differences in slopes are not taken into account. Therefore, the computation of a difference curve ensures consistent calculation of positive values of lysogenically produced viruses.

Finally, the lysogenic VP is translated into the percentage of lysogenic cells. The slope of the difference curve is equal to the lysogenic production rate, if there is a continuous increase of the lysogenically produced viruses during an experiment. If two peaks in viral abundance occur during the incubations, we add up the lysogenic VP in the time periods with a net increase. This yields the following formula for the proportion of lysogenic cells (see Table 1):

$$\%\ \text{of lysogenic cells} = 100 \times [(VP_{\text{lysogenic }1})/BS \times B_0] + \ldots + 100 \times [(VP_{\text{lysogenic }n})/BS \times B_0].$$

Demonstration of the program VIPCAL

We developed the program Viral Production Calculator (VIPCAL), which computes the lytic VP and the percentage of lysogenic cells based on data from a VRA. Parameters describing virus-induced mortality can also be calculated (Table 1).

The program, which is written in PHP scripts, is an online tool available at http://www.univie.ac.at/nuhag-php/vipcal.

Table 1. Computations used in the online tool Viral Production Calculator (VIPCAL).

Parameter	Formula	Related references
Calculated parameters based on the VRA		
Lytic		
Mean VP_{lytic}	$[(VDC_{max\,1} - VDC_{min\,1})/(t_{max\,1} - t_{min\,1}) + \ldots + (VDC_{max\,n} - VDC_{min\,n})/(t_{max\,n} - t_{min\,n})]/n$	This study
% of lytically infected cells	$100 \times [(VP_{lytic\,1})/BS \times B_0] + \ldots + 100 \times [(VP_{lytic\,n})/BS \times B_0]$	Jiang and Paul (1994); Weinbauer and Suttle (1996); Weinbauer *et al.* (2002); Winter *et al.* (2004)
Lysogenic		
% of lysogenic cells[a]	$100 \times [(VP_{lysogenic\,1})/BS \times B_0] + \ldots + 100 \times [(VP_{lysogenic\,n})/BS \times B_0]$	Jiang and Paul (1994); Weinbauer and Suttle (1996); Tapper and Hicks (1998); Weinbauer *et al.* (2002)
Other ecologically relevant parameters		
Lytic		
Lytic VP_{os} ($ml^{-1}\ h^{-1}$)	$VP_{lytic} \times (B_{os}/B_0)$	Wilhelm *et al.* (2002)
Lysis rate of bacteria (cells $ml^{-1}\ h^{-1}$)	Lytic VP_{os}/BS	Hewson and Fuhrman (2007b)
% of bacterial production lysed[b]	Lysis rate of bacteria/BSP_{os}	Hewson and Fuhrman (2007b)
% of bacterial loss per day	Lysis rate of bacteria $\times$ $100/B_{os} \times 24$	Hewson and Fuhrman (2007b)
Viral turnover time (h^{-1})	Lytic VP_{os}/V_{os}	Wilhelm *et al.* (2002)
DOC release (g C $ml^{-1}\ h^{-1}$)[c]	Lysis rate of bacteria $\times$ elemental carbon content of a bacterium	
DON release (g N $ml^{-1}\ h^{-1}$)[c]	Lysis rate of bacteria $\times$ elemental nitrogen content of a bacterium	

a. Based on the assumption that the burst size is the same in lytic and lysogenic infected cells.
b. Also termed virus-mediated mortality (VMM).
c. Release includes virus particles.
VP_{lytic}: lytic viral production from the experiment. VDC_{max}, VDC_{min}: maximal (max) and minimal (min) viral direct counts in a time period with a net increase; index *n* denotes the number of peaks. t_{max}, t_{min}: time period with a net increase in viral direct counts; index *n* denotes the number of peaks. $VP_{lytic\,1}, \ldots, VP_{lytic\,n}$: lytic viral production from the experiment; index *n* denotes the number of peaks. BS: burst size; it is assumed that the rate of viruses produced per cell is not affected by dilution. B_0: bacterial abundance at the beginning of the experiment. $VP_{lysogenic\,1}, \ldots, VP_{lysogenic\,n}$: lysogenic viral production from the experiment; index *n* denotes the number of peaks. Lytic VP_{os}: lytic viral production in the original sample. B_{os}: bacterial abundance in the original sample. BSP_{os}: bacterial secondary production in the original sample. V_{os}: viral abundance in the original sample. DOC: dissolved organic carbon. DON: dissolved organic nitrogen.

The main advantage of our method lies in its universal applicability to all possible outcomes of a VRA experiment.

Here, we demonstrate the application of the program for calculating lytic VP and the proportion of lysogenic cells in an environmental sample (Fig. 2). After starting the program, you can choose between two modes, a demo that introduces the program based on an example, and another for the input of new data. When choosing 'new data', the input field appears (Fig. 2A) and the sampling times have to be entered first. After entering each data point, press the enter button. Then enter the viral abundances – incubated with and without mitomycin C – for each sampling point of the experiment. Note that for correct calculation using VIPCAL, the VDC values must be expressed in 10^6 ml^{-1}. Replicates of viral abundance determinations can be entered. Separate each value by a semicolon. For further calculations, the program uses the mean viral abundance of each sampling point. Entering only the VDC without mitomycin C yields only lytic VP. Enter the following parameters to compute the percentage of lytically infected cells, lysis rate of bacteria, percentage of bacterial production lysed (also often called virus-mediated mortality), proportion of bacterial loss per day, viral turnover time and dissolved organic carbon and nitrogen release: bacterial abundance at the beginning of the VRA (in 10^6 ml^{-1}; replicates of bacterial abundance counts can be entered); viral and bacterial abundance (in 10^6 ml^{-1}), bacterial secondary production (in 10^6 cells ml^{-1} h^{-1}), the burst size in the original sample, as well as the carbon and nitrogen contents of a bacterial cell (e.g. Lee and Fuhrman, 1987; Simon and Azam, 1989; Fukuda *et al.*, 1998; Gundersen *et al.*, 2002). You can enter a variety of burst sizes. Separate each burst size by a semicolon. After entering all the data, press the pushbutton 'compute', and VIPCAL calculates the lytic and lysogenic VP of the respective experiment. When the results appear on the applet window, the mean lytic VP and the percentage of lytically infected cells are calculated based on the VRA (Fig. 2B). If you press the 'plot-button', a graphic representation of the two experimental curves (with and without mitomycin C) is provided, along with the computed difference curve, which corresponds to the lysogenically produced viral abundances (Fig. 2C). Based on the VRA the percentage of lysogenic cells is computed. Calculating the proportion of lysogenic cells provides information on whether the investigated environment favours the lysogenic life cycle or not. Note, however, that manipulations during the VRA may alter the proportions within the bacterial community. The calculation of the percentage of lysogenic cells is based on the assumption that the burst size is the same in lytically and lysogenically infected cells. Furthermore, not all lysogens can be induced with mitomycin C; therefore, this method most likely estimates the minimum percentage of bacteria in a community that are lysogens (Ortmann *et al.*, 2002).

The necessary manipulation steps in a VRA often reduce the initial bacterial abundance in the incubation. Therefore, VP estimates for the original environment should be corrected for the loss of bacteria between the original water and the incubation (Wilhelm *et al.*, 2002; Winget *et al.*, 2005). In VIPCAL, lytic VP rates can be corrected by the loss ratio of the bacterial abundance in the incubations at the beginning of the experiment. With this correction, the lytic VP has additional relevance for an investigated environment. If you enter the parameters 'bacterial abundance at the beginning of the VRA' and 'viral and bacterial abundance', their 'secondary production' and the 'burst size' in the original sample, as well as 'carbon and nitrogen contents of a bacterial cell', then VIPCAL also calculates the percentage of lytically infected cells, lysis rate of bacteria, percentage of bacterial production lysed, proportion of bacterial loss per day, viral turnover time, and dissolved organic carbon and nitrogen release. When mousing over the calculated parameter, a pop-up window appears containing the respective equation (Fig. 2B).

If you enter more than one burst size, all the parameters are first calculated based on the first entered burst size. Pressing the 'next burst size-button' calculates the values based on the second entered burst size and so on.

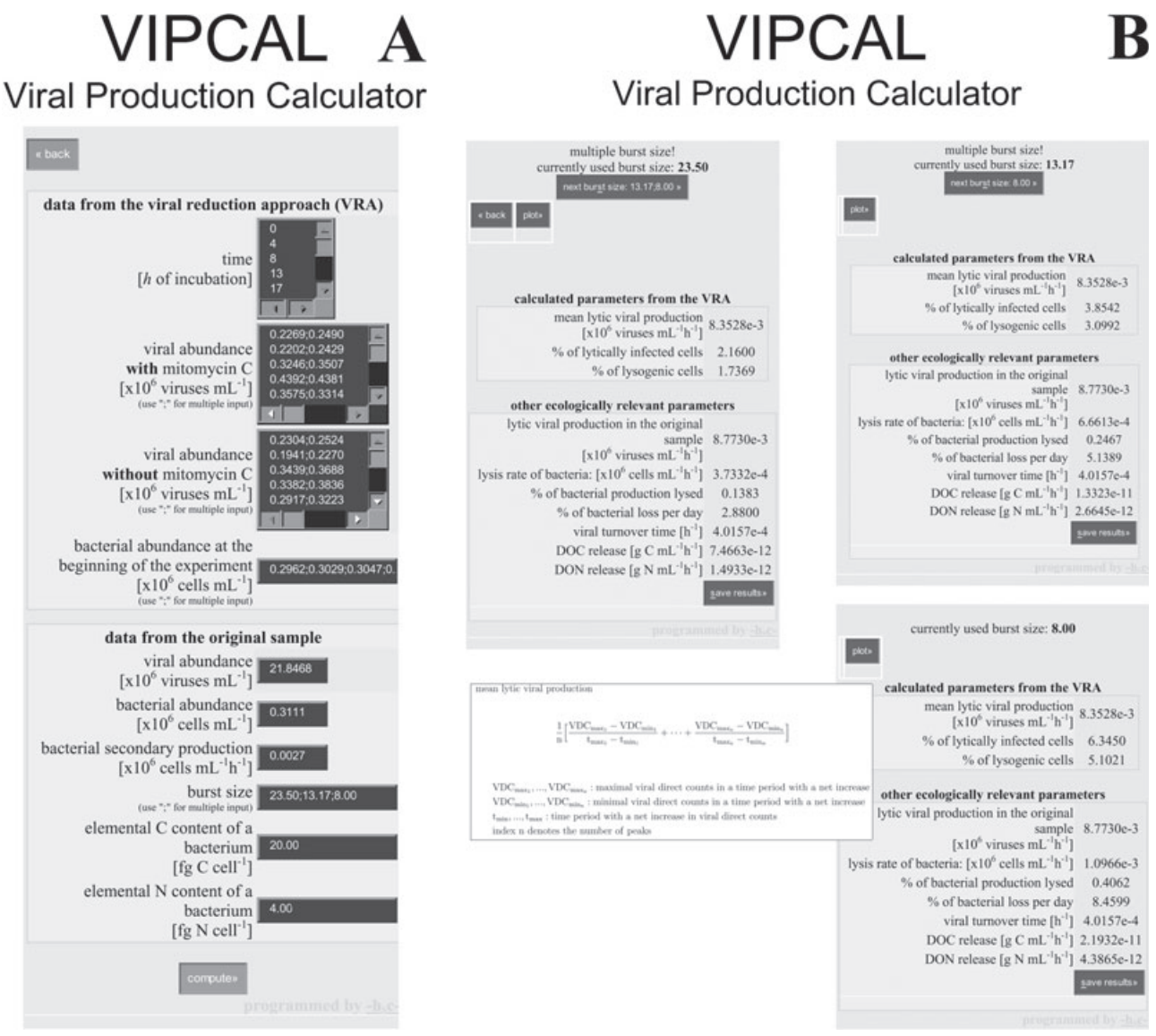
All entered parameters are saved in the input field (depending on the IP address of the computer). If you want to change a parameter, simply move back to the input field, change the specific value, and re-press 'compute'. All results are saved as csv (comma separated values) and can easily be exported into an Excel file.

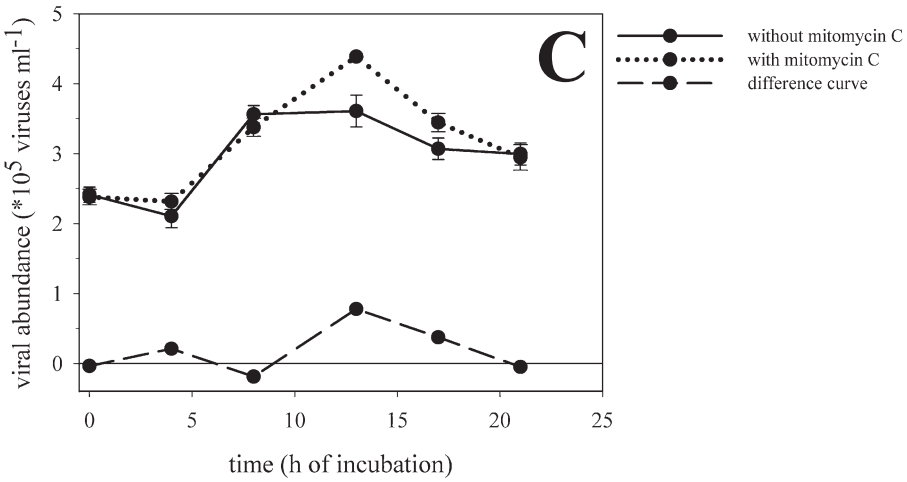
Although VIPCAL was developed to investigate lytic VP and lysogeny in heterotrophic bacteria, this program can, at least in principle, also be used to calculate viral parameters for infection of Cyanobacteria such as *Synechococcus* spp.

Conclusions

VIPCAL quantifies lytic and lysogenic VPs in a VRA and estimates the percentage of lysogenic cells. It can also calculate various viral parameters such as the percentage of lytically infected cells, lysis rate of bacteria, percentage

Fig. 2. Screen shot of the online tool Viral Production Calculator (VIPCAL). Input (A), output and pop-up (B) windows, data and graphic representation of curves (C) of the applet VIPCAL. VIPCAL calculates and plots the development of viral abundances (with and without mitomycin C) during the time of incubation (in hours) of a viral reduction approach (VRA). Calculated lytic and lysogenic viral production and various other viral parameters for an environmental sample are presented. The graph (C) shows the averages of the duplicate incubations. The error bars represent the range of the duplicate incubations. If the error bars are absent, they are smaller than the width of the symbol.





of bacterial production lysed, proportion of bacterial loss per day, viral turnover time and dissolved organic carbon and nitrogen release. This helps to characterize an environment based on the impact of lytic and lysogenic life cycles.

Using VIPCAL helps avoid differences between various studies or laboratories in calculating viral parameters, which facilitates the interpretation of results. Continuing methodological improvement such as the quantification of lytic and lysogenic viral pathways reveals more about the impact of viral infection on prokaryotes in aquatic ecosystems.

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