

ORIGINAL ARTICLE

The virus's tooth: cyanophages affect an African flamingo population in a bottom-up cascade

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Trophic cascade effects occur when a food web is disrupted by loss or significant reduction of one or more of its members. In East African Rift Valley lakes, the Lesser Flamingo is on top of a short food chain. At irregular intervals, the dominance of their most important food source, the cyanobacterium *Arthrospira fusiformis*, is interrupted. Bacteriophages are known as potentially controlling photoautotrophic bacterioplankton. In Lake Nakuru (Kenya), we found the highest abundance of suspended viruses ever recorded in a natural aquatic system. We document that cyanophage infection and the related breakdown of *A. fusiformis* biomass led to a dramatic reduction in flamingo abundance. This documents that virus infection at the very base of a food chain can affect, in a bottom-up cascade, the distribution of end consumers. We anticipate this as an important example for virus-mediated cascading effects, potentially occurring also in various other aquatic food webs.

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Introduction

Viruses are the most pervasive biological entities in aquatic ecosystems, the majority being bacteriophages (Suttle, 2007). Abundances of planktonic viruses are reported to commonly range between 10^4 (likely an underestimation due to bad storage conditions) and 10^8 ml^{-1} , being generally higher in freshwater than in marine systems. Peak values were found in very productive estuaries and lakes (Peduzzi and Luef, 2009). Exceptionally high virioplankton abundance (up to $2 \times 10^9 \text{ ml}^{-1}$) has been documented in the alkaline, hypersaline Mono Lake, California (Brum *et al.*, 2005). Typically, virus replication rates increase in conjunction with higher host growth rates (Suttle, 2007). Beyond the potential to influence and control abundance and diversity of heterotrophic prokaryote hosts, viruses apparently infect a significant proportion of the photoautotrophic plankton community as well (Wommack and Colwell, 2000; Weinbauer, 2004; Suttle, 2007). Particularly cyanophages have been shown to cause mortality in important members of the phytoplankton (Suttle and Chan, 1994).

The Lesser Flamingo, *Phoeniconaias minor*, is the dominating and characteristic bird species of alkaline-saline lakes and pans of East Africa (Krienitz and Kotut, 2010) and classified as 'near-threatened' (IUCN Red List, 2012). The dense population of these pinkish waterfowl (1.5–2.5 million in Eastern Africa, which is around 75% of the worldwide occurrence) has been rated since decades as one of the most significant wildlife spectacles worldwide (Jenkin, 1929). Flamingos are filter feeders of photoautotrophic algal primary producers, putting them on top of a short and direct food chain (Vareschi and Jacobs, 1985). The Lesser Flamingo relies on the very fast-growing alkaliphilic cyanobacterium *Arthrospira fusiformis* as most important food source. Fluctuations of this resource are reported as the overwhelming factor influencing Lesser Flamingo distribution (Vareschi and Jacobs, 1985; Krienitz and Kotut, 2010). However, the typically dense (up to $>700 \text{ mg l}^{-1}$ fresh weight; Krienitz and Kotut, 2010) population of *A. fusiformis* collapses unpredictably. This is thought to be the major cause for episodes of disappearance or significant irregular mass movements of flamingos between the lakes (Krienitz and Kotut, 2010). Causes for the sudden *A. fusiformis* breakdowns have remained a matter of speculation.

At latest Eisenberg's inspiring book 'The Wolf's Tooth' (Eisenberg, 2010) cemented awareness in the scientific community about how trophic cascades can impact food webs. Trophic cascades have been documented for all of the world's major biomes,

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in terrestrial and aquatic systems, from the poles to the tropics. For example, the anthropogenically mediated loss of apex consumers is discussed as leading to extensive top-down cascading effects in marine, terrestrial and freshwater ecosystems worldwide (Eisenberg, 2010; Estes *et al.*, 2011). Whereas top-down forcing is well documented (Shurin *et al.*, 2002), bottom-up cascading has been less discussed. This phenomenon occurs if, for example, a primary producer is removed, resulting in diminished population sizes through the community. The effect of trophic cascades is assumed to be stronger in aquatic than in terrestrial food webs (Shurin *et al.*, 2002). On the one hand, this may be due to the higher mass-specific biomass production in phytoplankton and benthic algae compared with terrestrial vascular plants (Cebrian, 1999); on the other hand, herbivores consume on average a threefold greater amount of lentic plankton than plant consumption in terrestrial food webs (Cyr and Pace, 1993). The consensus is that cascade effects are more severe with decreasing food web complexity (Strong, 1992).

We know that nomadic movements or disappearance of the Lesser Flamingo are linked to changes in food quantity and quality (Tuite, 1979; Owino *et al.*, 2001; Krienitz and Kotut, 2010; Zaccara *et al.*, 2011). We hypothesized that viruses (as most abundant biological entities) are capable of mediating a bottom-up cascade (*sensu* Hunter and Price, 1992), thus being responsible for the frequent breakdowns in *A. fusiformis* biomass and, as a consequence, for the drastic drop in flamingo numbers at Lake Nakuru. We document a first example for such a virus-mediated bottom-up cascade in this short food chain from photoautotrophic prokaryotes to birds.

Materials and methods

Sampling location

Lake Nakuru is a shallow, alkaline–saline water body situated in Lake Nakuru National Park, Kenya. Its size covers 44 km², the average depth is 2.5 m, which can reach a maximum level of 4.5 m (<http://www.worldlakes.org/>). It is mainly recharged by rainfall falling directly on the lake. It also occasionally receives inflows from the three seasonal surface streams Njoro, Makalia and Nderit, as well as from the Baharini Spring at the northern end. Municipality sewage is also discharged into the lake. The lake is world-famous for its waterfowl population. Selected limnological characteristics (temperature, soluble reactive phosphorus, pH, conductivity, salinity, total alkalinity as well as overall bacterial and virus abundance) were monitored during the investigation period. Further and more detailed information on hydrochemistry and lake characteristics was reported earlier (Oduor and Schagerl, 2007; Jirsa *et al.*, 2013).

Sample collection

Sampling was done in the central part of the lake at the coordinates 00° 21.387' S, 036° 05.519' E. Surface samples (0.5–1.0 m) were taken weekly between 7 January and 27 October 2009. Samples for counting heterotrophic bacteria, viruses and phytoplankton were taken with a Schindler trap and immediately fixed with glutaraldehyde to a final concentration of 2%. Samples for cyanophage screening within *A. fusiformis* cells by TEM (transmission electron microscopy) were collected with a plankton net (mesh size 30 µm) and fixed as above.

Water temperature was measured by data loggers (HOBO, Onset Computer Corporation, Bourne, MA, USA) with logging intervals of 5 min. Salinity, electrical conductivity and pH were measured on site using a multiprobe (WTW Multi 340i Wissenschaftlich Technische Werkstätten Weilheim, Germany). Soluble reactive phosphorus was analyzed by a modified standard method (Clesceri *et al.*, 1998), considering the high buffer capacity of the water.

Counting of viruses and heterotrophic bacteria in the water column, screening for cyanophage infection, *A. fusiformis* biomass

For heterotrophic bacteria and total virus counts, 0.5–1 ml sample water was filtered within 1–2 h on Whatman Anodisc filters (pore size 0.02 µm; 25 mm diameter), stained with SYBR GREEN 1 and counted with an epifluorescence microscope (Motic BA 4000, Motic, Hong Kong, China) (Noble and Fuhrman, 1998). To estimate the *A. fusiformis* biovolume, water samples were fixed with 5% formaldehyde. Filaments were classified into three groups based on their morphology and enumerated and measured under an inverted microscope (Nikon Diaphot, Nikon, Japan) (Utermöhl, 1958). Filament dimensions of the morphology groups, which included height of coil, coil diameter and number of coils were measured at × 400 magnification. Each morphological variable was measured on at least 50 filaments. For total biovolume calculation, the number of filaments was multiplied by the respective filament volume. For more details, see Kaggwa *et al.* (2013). For conversion of cell volume to biomass in milligram, a conversion factor for density of 1 was used according to Wetzel and Likens (1991).

To screen for visible cyanophage infection (frequency of visibly infected cells; Proctor and Fuhrman, 1990; Binder, 1999) in *Arthrospira* cells, the material was concentrated via gentle centrifugation and then preserved in sodium cacodylate buffer after three rinsing steps with buffer solution (Glauert, 1975). The filaments were then transferred into 2 ml Eppendorf vials and stored in the dark at 4 °C until further analysis. For TEM preparation, the samples were rinsed with a buffer solution to get rid of interfering dissolved organic substances. The residue was then deposited in

glutaraldehyde (2.5%) for at least 15 h, rinsed five times (each 10 min) with double-distilled water and transferred into osmium tetroxide (2%) for 2 h. After post-fixation and three more rinsing steps (double-distilled water, each step 20 min), dehydration was done in 100% acetone (two times, 15 min each). Samples were then transferred into Agar Low Viscosity Resin (Agar Scientific, Stansted, Essex, UK) embedding medium, diluted 1 + 1 with acetone and left for 3 h to facilitate infiltration, which was assisted by a specimen rotator. After transfer into pure embedding medium, the samples were left for 4 h and then the supernatant resin was replaced. The sample was then placed for polymerization at 40 °C in a cabinet dryer overnight followed by 24 h at 65 °C.

The slug was trimmed and ultra-thin sections were performed (70 nm; diamond knife, DiATOME, Biel-Bienne, Switzerland; Ultramicrotome Ultracut E, Reichert Jung, Austria). To ensure the observation of different filaments, the slug was trimmed again by additional 25 µm after every five sections and another series was cut. Ultra-thin sections were placed on copper grids and contrasted with uranyl acetate (0.5%) for 30 min followed by lead citrate (3%) for 7 min. The samples were analyzed in a TEM Zeiss 902. In total, 4068 cells were sliced and the ultrathin sections were inspected for visible signs of infection and photographed. On average, 131.2 cells were sliced per each sampling date when *A. fusiformis* was present. We also considered areas within the host cells exhibiting disintegrated cytoplasm structure (Figures 2a and b), which contained only few or no virus particles as visible signs of infection (these two categories are distinguished in Figure 1a). This is based on the observation that visible virus particles were always associated with the same disintegrated cytoplasm structure. Ultra-thin sections, however, can eventually cut through the fringe of a viruses-infected area, thus resulting in empty or only few (<5) virus particle-containing sectors. Therefore, we decided to include this information to the results, but distinguished between these two categories. The frequency of visible infected cells is expressed as % of the total number of inspected cells (Proctor and Fuhrman, 1990; Binder, 1999).

Flamingo censuses

The development of the Lesser Flamingo abundance at Lake Nakuru during the investigation period was followed by estimating total numbers, applying ground-based line transects along the lake shoreline (Lloyd *et al.*, 1998).

Results

Table 1 presents selected limnological characteristics and overall bacterial and virus abundance of

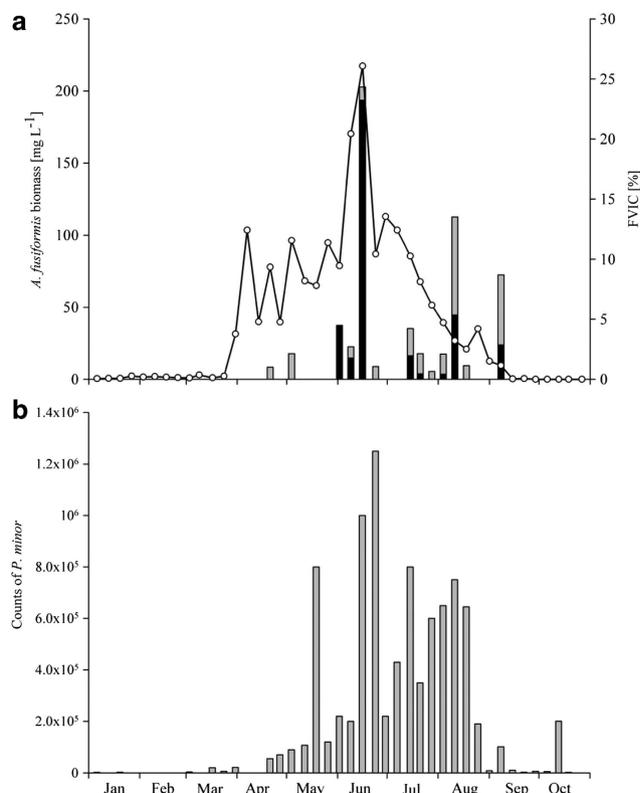


Figure 1 Development of food algae, cyanophage infection and flamingo abundance. (a) *A. fusiformis* biomass (open circles), frequency of visible infected cells (FVIC; bars); black bars, percentage of the number of cells with areas containing at least five visibly developed cyanophages; grey bars, percentage of cell numbers containing infection areas with <5 or no visibly developed cyanophages. (b) Flamingo abundance observations at Lake Nakuru between January and October 2009.

Table 1 Selected variables for characterising Lake Nakuru during the entire investigation period

	Mean	Range
Temperature (°C)	25.1	21.6–30.3
pH	10.1	9.5–10.7
Conductivity (mS cm ⁻¹)	53.4	30.6–96.2
Salinity (‰)	35.7	19.2–62.1
Total alkalinity (meq l ⁻¹)	736	344–1482
SRP (µg l ⁻¹)	1361	74–5458
Viruses (ml ⁻¹)	4.8×10^9	1.2×10^9 – 7.0×10^9
Heterotrophic bacteria (ml ⁻¹)	1.8×10^8	5.0×10^7 – 3.1×10^8
VBR	27.1	10.0–48.6

Abbreviations: SRP, soluble reactive phosphorus; VBR, virus to bacteria ratio

this lake during the investigation period. The values underline the extreme conditions in this alkaline-saline environment. Further, the samples revealed very high numbers of viruses (total counts of the viral community) of up to 7.0×10^9 ml⁻¹. Also heterotrophic bacterial abundance was high; the virus-to-bacteria ratio fluctuated but remained in the same order of magnitude.

During the study period, an increase and a subsequent breakdown of both *A. fusiformis*

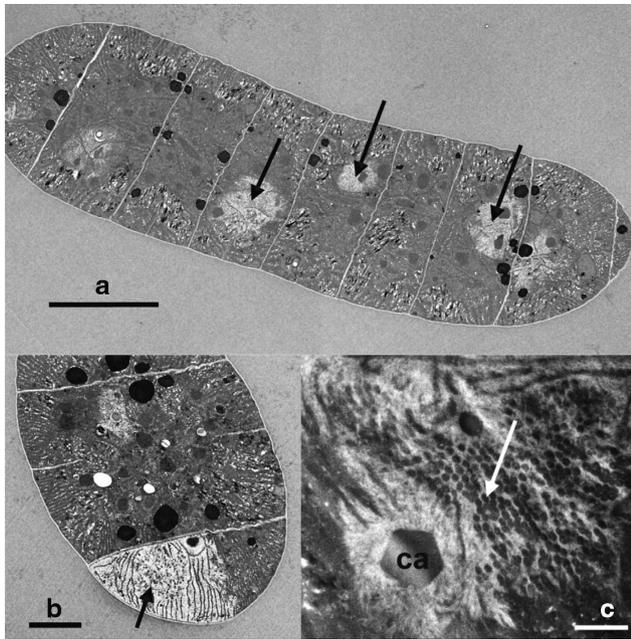


Figure 2 Visible signs of cyanophage infection in *A. fusiformis* cells. (a) longitudinal section through a coil of a cell filament; arrows point to infected areas, which appear bright and disarranged, scale bar 5 μm ; (b) close up of an infected area (arrow) with signs of already assembled capsids, scale bar 2 μm ; (c) area containing already visibly developed cyanophages (white arrow); the large polyhedral particle (ca) represents a carboxysome; scale bar, 0.5 μm .

biomass and Lesser Flamingo abundance occurred (Figures 1a and b). *A. fusiformis* biomass fluctuated between low values of 0.44 mg l^{-1} in January and a maximum of 217.3 mg l^{-1} in June 2009. Here, at peak phytoplankton biomass, almost 100% of the total algal biomass was comprised by *A. fusiformis* (Kaggwa *et al.*, 2013). After this maximum, *A. fusiformis* biomass dropped again (with a slight recovery in late June and August) to undetectable levels in October.

During the crash of *A. fusiformis*, visible signs of cyanophage infection were detected by TEM analyses (Figure 2). The highest frequency of visible infection (24.3% of inspected cells) occurred at peak abundance of *A. fusiformis* host cells, which clearly marked the onset of this biomass breakdown. From early June until the complete vanishing of *A. fusiformis*, almost each sampling revealed a high percentage (on average 5.9%) of visible infections (Figure 1a).

Low flamingo numbers of around only 100 individuals were detected at the beginning of the year, followed by an increase in April, reaching a dense population of >1 250 000 in June 2009 (Figure 1b). This pattern coincided with food availability of *A. fusiformis*. Together with the breakdown of the algal biomass, a drastic decrease in flamingo abundance was observed, with a lowest number of ~ 1500 individuals in October. The correlation between *A. fusiformis* biomass and

P. minor numbers was significant ($r=0.70$, $P=0.001$, $n=38$; Spearman, two-tailed).

Discussion

Along with its specific chemical and biological features, Lake Nakuru is also extreme in its virioplankton abundance. To our knowledge, the samples revealed the highest numbers of viruses ever reported in any natural aquatic environment. This strongly supports the significant role that viruses may have in such an environment, for example, as important mechanism of mortality in prokaryotes.

Very recently, a cyanophage was described that infects *Arthrospira platensis* in mass cultures (Jacquet *et al.*, 2013). In our study, the frequency of visible infected cells between June and September was high (compare Binder, 1999). Considering that only a certain fraction is actually in the visible state of infection (Proctor and Fuhrman, 1990; Binder, 1999), most likely an even larger proportion of the population was infected during this period. A virus-mediated impact on the *A. fusiformis* host population can be assumed by several causes. Two viral cycles are important in aquatic habitats, lysogeny and lytic infection, the latter being strongly related to host abundance (Weinbauer, 2004). Clearly, the host cell density and the linked infection probability were very high, not only at the June biomass peak. Furthermore, when considering induction as a significant mechanism, the transition from a lysogenic state to host cell lysis is often linked to various types of inducing agents. Some of these might be of particular importance in the investigated environment, such as sunlight and UV radiation, temperature changes, aromatic compounds and changes in ion contents (Jiang and Paul, 1996; Weinbauer, 2004). Water temperature was apparently not important because it fluctuated only moderately during the investigation period (see Table 1). Salinity, however, almost doubled during the cyanoprokaryote breakdown from June to October, from 32‰ to 62‰. We did not collect data on UV exposure, but it could have been important especially during calm periods, when buoyant *A. fusiformis* filaments develop scums on the lake surface.

In addition, other factors can be responsible for dynamic population oscillations in such a virus–host system. There is evidence that the presence of bacteriophages is crucial in generating genetic diversity such as strains and ecotypes (Rodríguez-Valera *et al.*, 2009). For a major component of marine picoplankton (*Synechococcus*), genetic diversity has been linked to interactions with co-occurring phages, making phages determinants for the abundance, decline as well as succession of different clones (Mühling *et al.*, 2005). Such results indicate that both the abundance and genetic

diversity of cyanophages co-varied with their hosts. This is consistent with cyanophage infection as a major controlling factor in phytoplankton successions (Suttle and Chan, 1994; Wang and Chen, 2004; Mühling *et al.*, 2005). We assume that the irregular and unpredictable collapses of *A. fusiformis* may also be the result of dynamic interactions between resistant and non-resistant host strains and co-occurring, genetically diverse cyanophage populations.

From our investigation we conclude that, at Lake Nakuru, a virus-mediated loss of the most important food source triggered the collapse in *P. minor* abundance. The consumption activity of the flamingos on *A. fusiformis* is unlikely to be responsible for the stochastic breakdowns of the algal biomass. At a medium phytoplankton biomass at Lake Nakuru (145 mg l^{-1}), a flamingo population of 1 million birds was calculated to extract up to 60 t algal mass per day (Vareschi, 1978; Krienitz and Kotut, 2010). On the basis of the average lake volume, the entire lake then harbors some 13×10^4 t of *A. fusiformis*, thus only 0.46% of the standing stock would be consumed per day. Further, *A. fusiformis* tolerates high alkalinity, salinity and elevated temperatures of $> 35^\circ \text{C}$ (Vonshak and Tomaselli, 2000). During the investigation period, all these parameters remained, together with soluble reactive phosphorus, within a range that does not limit *A. fusiformis* growth (see Table 1). Moreover, top-down control on the *A. fusiformis* population by zooplankton was recently excluded as a reason for biomass breakdowns in our study environment (Burian *et al.*, 2013), whereas a virus-mediated control was not considered in any previous study. On the basis of the decrease of *A. fusiformis* biomass during 8 days after the biomass peak in mid of June, $> 60\%$ of the algal biomass has disappeared, which resembles a mortality rate of $16.3 \text{ mg l}^{-1} \text{ d}^{-1}$. However, this mortality rate is likely even an underestimation as it does not consider the ongoing algal production. Under continuing infection pressure in the following period the complete *A. fusiformis* food biomass vanished from the environment, thus being likely the major cause for disappearance also of the flamingos.

Our results point to a previously undocumented virus attack on the highly specialized algal diet of the Lesser Flamingo. Moreover, this infection is likely responsible for a collapse in flamingo numbers in a bottom-up cascade. The adverse environment facilitates excessive growth of only a few extremophiles. The result is low diversity and short food chains, which are apparently highly susceptible to various kinds of episodic disturbance events like virus attack. We suggest more in-depth investigations of the role of viruses in trophic interactions and cascades, and in different types of food webs. We propose that virus-related processes should be more comprehensively incorporated in ecological theory, thus adding complexity to trophic cascade concepts.

Conflict of Interest

The authors declare no conflict of interest.

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