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The East African Rift Valley Lakes Bogoria and Nakuru sometimes host around 75% of the world population of lesser flamingos *Phoeniconaias minor*. In this area, mysterious flamingo die-offs have occupied researchers for four decades. Recently, cyanobacterial toxins came into the fore as a possible explanation for mass mortalities because the main food source of lesser flamingos is the cyanobacterium *Arthrospira fusiformis*. We took weekly samples from July 2008 to November 2009 from Lakes Nakuru and Bogoria and analyzed them by high performance liquid chromatography for microcystins. Monthly, samples were cross-checked using protein phosphatase inhibition assays with lower detection limits and additionally screened for polar toxins. During our study period, three flamingo die-offs occurred at L. Bogoria and we were able to analyze tissues of 20 carcasses collected at the shoreline. No cyanotoxins were detected either in plankton samples or in flamingo tissues. Accordingly, other reasons such as food composition or bird diseases played a key role in the observed flamingo die-offs.

The cyanobacterium *Arthrospira fusiformis* is a well-known dietary supplement because of its high content of essential amino acids and fatty acids, vitamins, minerals and antioxidant functions (Belay et al. 1993, Capelli and Cysewski 2010). In its natural habitat, i.e. tropical alkaline–saline water bodies, *A. fusiformis* commonly plays a key role in the food web as the main photoautotrophic primary producer (Vareschi and Jacobs 1984, Oduor and Schagerl 2007). We focused our research on the Kenyan soda lakes Bogoria and Nakuru, where *A. fusiformis* is the main food source for lesser flamingos *Phoeniconaias minor* (Vareschi 1978).

Die-offs of lesser flamingos at some East African rift valley lakes have attracted researchers interests for four decades (Table 1). They are a threat to the economic and conservation status of the otherwise impoverished regions whose lakes host these birds. Possible causes that have been cited involve habitat degradation (Krienitz and Kotut 2010), infectious diseases (Sileo et al. 1979, Kock et al. 1999) and pollution through heavy metals and pesticides (Greichus et al. 1978, Kairu 1996). Additional suggestions include changes in food quantity and diet quality (Motelin et al. 2000, Ndetei and Muhandiki 2005, Krienitz and Kotut 2010, Kaggwa et al. 2013) and toxic cyanobacteria (Ballot et al. 2004a, b, Krienitz et al. 2005, Koenig 2006, Lugomela et al. 2006).

To date, the ultimate causes for the die-offs are still speculative in most cases, because prompt action is needed to collect material. As this phenomenon usually occurs in remote areas and carcasses are removed by scavengers within a few days, this is rarely possible. In July 2008, a flamingo die-off was observed at L. Bogoria (Krienitz and Kotut 2010) and in 2009, two smaller but noticeable flamingo die-offs (March and August) occurred at the same lake (Fig. 1, Table 1). During our weekly sampling intervals from July 2008 to November 2009, we had the opportunity to screen the lake water for cyanotoxins. This approach was based on reports of toxin-producing cyanobacteria in the hot springs around L. Bogoria and in two flamingo carcasses (Krienitz et al. 2003, Metcalf et al. 2013). Harper et al. (2003), however, argued that those observations were not fully representative of the whole lake as the hot springs represent only a small proportion of the available drinking water. Very recently, Metcalf et al. (2013) detected neurotoxic amino acids in flamingo feathers, which were collected during a sudden mass mortality in 2003, so cyanotoxins must not be precluded as a possible explanation for the recent die-offs.

Methods

The lakes Nakuru and Bogoria sometimes host 75% of the world population of lesser flamingos *Phoeniconaias minor* (Nasirwa 2000, Zaccara et al. 2011), are amongst the Kenyan top tourist attractions and are classified as National Park

Figure 1. Flamingo die-off at the shore of Lake Bogoria in July 2008. Within one week, about 30 000 out of 150 000 flamingos died.

(Nakuru) and Ramsar site (Bogoria). The surface area of L. Nakuru (00°S, 36°E; 1756 m a.s.l.) between 36 and 49 km² depends on precipitation, evaporation and human use such as irrigation and varies highly throughout the year. The lake has a maximum depth of about 1 m and is daily mixed. The surface area of L. Bogoria (00°N, 36°E; 990 m a.s.l.) is usually about 34 km², but extending during distinct rainy seasons especially in the northern area. With a maximum depth of 10.2 m (Hickley et al. 2003), it is stratified over longer periods (McCall 2010).

We took weekly samples at both lakes. Algal composition and biovolume were determined using lake water samples fixed with formalin to a final concentration of 4%. Taxa were identified down to the lowest possible level and enumerated using an inverted microscope at 100× and 200× magnification (for *Arthrospira* and *Anabaenopsis*) and 400× for other taxa. Taxon specific biovolumes were estimated using geometric formulae of shapes similar to the respective phytoplankton cells. At least thirty cells for each taxon identified were measured to give the average size and biovolume. For conversion of cell volume into biomass, a conversion factor of 1 was used.

A 30-μm-mesh plankton net was used to collect particle sizes that are usually retained by the filter of the lesser flamingo bill and swallowed. Samples were hauled from the shoreline at the central sampling station of the respective lake in weekly intervals. The samples were transported cooled to the Egerton laboratory, immediately filtered through glass-fiber filters and then the filters containing algal biomass were dried and stored frozen until analysis. Prior to analysis, filters were cut into pieces, put in Eppendorf tubes and homogenized with a tissue grinder to assist cell burst and

toxin extraction. Extraction was done in 75% (w/v) aqueous methanol. The extracts were concentrated to dryness in a vacuum centrifuge and the residue redissolved in 300 μl of 50% aqueous methanol, vortexed and centrifuged at the highest level for 10 min. Finally, the clear supernatant was analyzed as described in Kurmayer et al. (2003) with high performance liquid chromatography/diode array detection (HPLC-DAD). A gradient of aqueous acetonitrile containing 0.05% triflouroacetic acid (TFA) was used at a flow rate of 1 ml increasing from 30 to 70% acetonitrile in 42 min. MCs with a limit of quantification (LOQ) of 100 ng l⁻¹ were identified by their retention times, co-chromatography with authentic standards (Sigma-Aldrich 33578 and CyanoBiotech), and their characteristic absorption spectra. Additionally, 13 dried filters of net samples were analyzed for microcystins in the Aberdeen laboratory for a crosscheck, (MC–LC, –RR, –YR, –LW and –LF; standards available from EnzoLife sciences, Lausen, Switzerland).

To check for traces of MCs, protein phosphatase-inhibitions assays (PPIA) (An and Carmichael 1994) with a detection limit of 10 ng l⁻¹ were performed in monthly intervals in 96-well plates; PP1 was used to hydrolyze p-nitrophenol-phosphate. If MC was in the samples, this reaction would have been inhibited and so no color difference would occur with time. The reaction results in a chromogenic product with an absorbance maximum at 405 nm. Plates were then measured by a plate reader.

During periods of die-offs in July 2008, March and August 2009, we additionally analyzed samples for polar toxins, such as cylindrospermopsin, anatoxin-a, anatoxin-a(S), saxitoxin and neosaxitoxin, in the Aberdeen laboratory by means of LC–TOF–MS (Waters Acquity ultra-high performance liquid chromatography coupled to a Xevo quadrupole time of flight mass spectrometer; standards: Cylindrospermopsin EnzoLife Sciences, Phenylalanine Sigma, Anatoxin-a Calbiochem, Saxitoxin and Neosaxitosin NRC Canada). Dried samples (50 mg) were extracted in 1 ml of methanol containing 0.1% TFA for 1 h with intermittent vortexing. An Atlantis hydrophilic interaction (HILIC) column (2.1 mm i.d. ×150 mm long; 5 μm particle size) was used for the separation; mobile phase consisted of acetonitrile (A) and MilliQ containing 0.5 mM formic acid and 0.2 nM ammonium formate (B). Separation was achieved using a gradient increasing from 2% B to 20% B over 7 min, followed by an increase to 70% B from 7 to 10 min at a flow rate of 0.3 ml min⁻¹. Data was acquired in positive ion electrospray scanning from m/z 50 to 600 with a scan time of 2 s and inter-scan delay of 0.1 s. Ion source parameters: capillary and sampling cone were 2.9 V and

Table 1. Reported flamingo mass-mortalities in the Rift Valley Lakes Nakuru and Bogoria from 1991 to 2009.

Year	No. of carcasses	Season	Lake	References
1991	40 000		Bogoria and Nakuru	unpublished data
1993	30 000	Sept–Nov	Bogoria and Nakuru	(Kock et al. 1999, Ndetei and Muhandiki 2005)
1995/1996	50 000		Bogoria and Nakuru	(Motelin et al. 2000, Krienitz et al. 2005)
1999/2000	30 000		Bogoria	(Krienitz et al. 2003)
2006	35 000	July, Aug	Nakuru	(Krienitz and Kotut 2010)
2008	30 000	July	Bogoria	(Krienitz and Kotut 2010), this study
2009	2000	March, Aug	Bogoria	this study

25 V respectively; desolvation temperature 300°C; source temperature 80°C. Cone gas and desolvation gas flows were 50 l h^{-1} and 400 l h^{-1}, respectively. Sodium iodide (2 μg μl^{-1} in 50/50 propan-2-ol/H$_2$O) was used as the calibrant with leucine-enkephalin (0.5 mg ml^{-1} in 50/50 methanol/Milli-Q) as the lockspray. Instrument control, data acquisition (centroid) and processing were achieved using MassLynx ver. 4.1. LOQ for cylindrospermopsin was 100 ng ml^{-1}, other polar toxins were determined on a qualitative basis due to lack of availability of accurate standards. Phenylalanine is always monitored as this is often misidentified as anatoxin-a.

In addition, material of flamingo carcasses (gizzard, muscle, liver and intestine) from the die-off in August 2009 was analyzed. Tissues were lyophilized, homogenized in a tissue grinder and then extracted according to the aforementioned protocol in 1 ml of methanol containing 0.1% TFA for 1 h with intermittent vortexing. Afterwards the samples were centrifuged at 15 000 g for 10 min. Then the supernatant was removed and used for microcystin and polar toxin analyses.

Results

During the survey, we observed three flamingo die-offs in the Bogoria area. The largest die-off occurred during July 2008, where about 30 000 birds deceased within one week (Fig. 1, Table 1). At this time, the lake hosted about 150 000 flamingos. Two smaller die-offs occurred during 2009 with around 2000 carcasses at the central shoreline area of the lake. During the second die-off in August 2009, we were able to gather different tissues from 20 flamingo carcasses (Table 2) and neither could detect MCs nor polar cyanotoxins.

Net plankton samples were analyzed in weekly intervals for MCs by HPLC and no MCs were detectable. The sensitive PPIA applied in monthly intervals confirmed the HPLC results (Table 2). We also were not able to detect cylindrospermopsin, anatoxin-a, anatoxin-a(S), saxitoxin and neosaxitoxin during the three die-off periods. Summarizing up, in none out of 156 samples analyzed with different methods, we were able to detect any cyanotoxins (plankton n = 136; flamingo-tissues n = 20).

During the study, also the algal composition and biovolume were monitored (Fig. 2). Cyanbacteria turned out to be the dominant group in both lakes. In L. Bogoria, *Arthrospira fusiformis* showed a more or less mono-specific dominance during the whole study period. L. Nakuru showed greater variation in the algal community composition

Table 2. Analyzed samples during the investigation. In none of the samples, cyanotoxins were detectable (LOQ HPLC > 100 ng l^{-1}; PPIA > 10 ng l^{-1}; LC/TOFMS > 10 μg l^{-1}).

Samples	HPLC	PPIA	LC/TOFMS
Lakes			
Bogoria central	68	17	13
Nakuru central	68	17	12
Flamingo tissues			
Gizzard	6		6
Muscle	9		9
Liver	2		2
Intestine	3		3

with also green algae and Cryptophyta being abundant. At certain times, *Arthrospira* blooms crashed and were replaced by eukaryotic taxa.

Discussion

Our surprising results contradict other studies (Krienitz et al. 2003, Ballot et al. 2004a, Metcalf et al. 2006, 2013), prompting a search for explanations for this discrepancy: 1) the occurrence of the main toxin producer *Microcystis* in such systems is still disputed. While some scientists proposed a link between *Microcystis* and flamingo die-offs (Ndetei and Muhandiki 2005, Stewart et al. 2008, Githaiga 2003), others challenged the growth of *Microcystis* in such alkaline-saline environments (Kotut and Krienitz 2011). Former *Microcystis* occurrences (Ndetei and Muhandiki 2005) were probably misidentified and may belong to *Anabaenopsis abijatae*, which at a first glance could be mixed up with *Microcystis* colonies. Even molecular techniques could not detect any phylotypes of *Microcystis* (Dadheech et al. 2009, Kotut and Krienitz 2011). 2) *Arthrospira fusiformis* and *A. maxima* are sold as food supplements and considered as non-toxic. Studies on the toxicity of the dominant cyanobacterium *A. fusiformis* revealed that a few clones produce MCs and anatoxin-a, but others not (Ballot et al. 2004a). Mussagy et al. (2006) considered three strains isolated from Mozambique and one from L. Nakuru; none of them produced toxins, nor could the mcyE gene be detected from the analyzed Mozambique clones. At least for our study period, the toxicity of *A. fusiformis* could be excluded as the net samples consisted of mainly cyanobacterial biomass with *A. fusiformis* being the dominant taxon. 3) No cyanotoxins could be detected in flamingo tissues collected during this study, which is in accordance to conclusions of Kock et al. (1999), who examined inner organs of 42 carcasses in 1993 and did not find any typical indications of cyanotoxins poisoning. As cyanotoxins have been detected in flamingo feathers even seven years after collection (Metcalf et al. 2013), we know that flamingos are exposed to these toxicants at least from time to time. It however seems unlikely that the ingested toxins were solely responsible for the die-offs, because unusual mortalities of other bird taxa and mammals frequenting the lakes in high numbers have never been recognized and also scavengers feeding on flamingo carcasses were obviously not affected.

What else could have caused die-offs during our sampling period? Interestingly, this phenomenon has been observed only at Lake Bogoria during our study. Both lakes Nakuru and Bogoria showed varying *A. fusiformis* biomass, which has been identified as driving force for flamingo movements (Kaggwa et al. 2013). The lakes, however, do differ somewhat. While Lake Bogoria was dominated by *A. fusiformis* throughout the sampling period, Lake Nakuru showed pronounced changes in the phytoplankton composition; especially after *Arthrospira* biomass crashes, shifts towards other algae taxa occurred (Kaggwa et al. 2013). The flamingo deaths in March and August 2009 corresponded with the highest bird densities at Lake Bogoria. Kaggwa et al. (2013) proposed that neither food quantity nor food quality in terms of carbohydrates, lipids and crude protein were likely
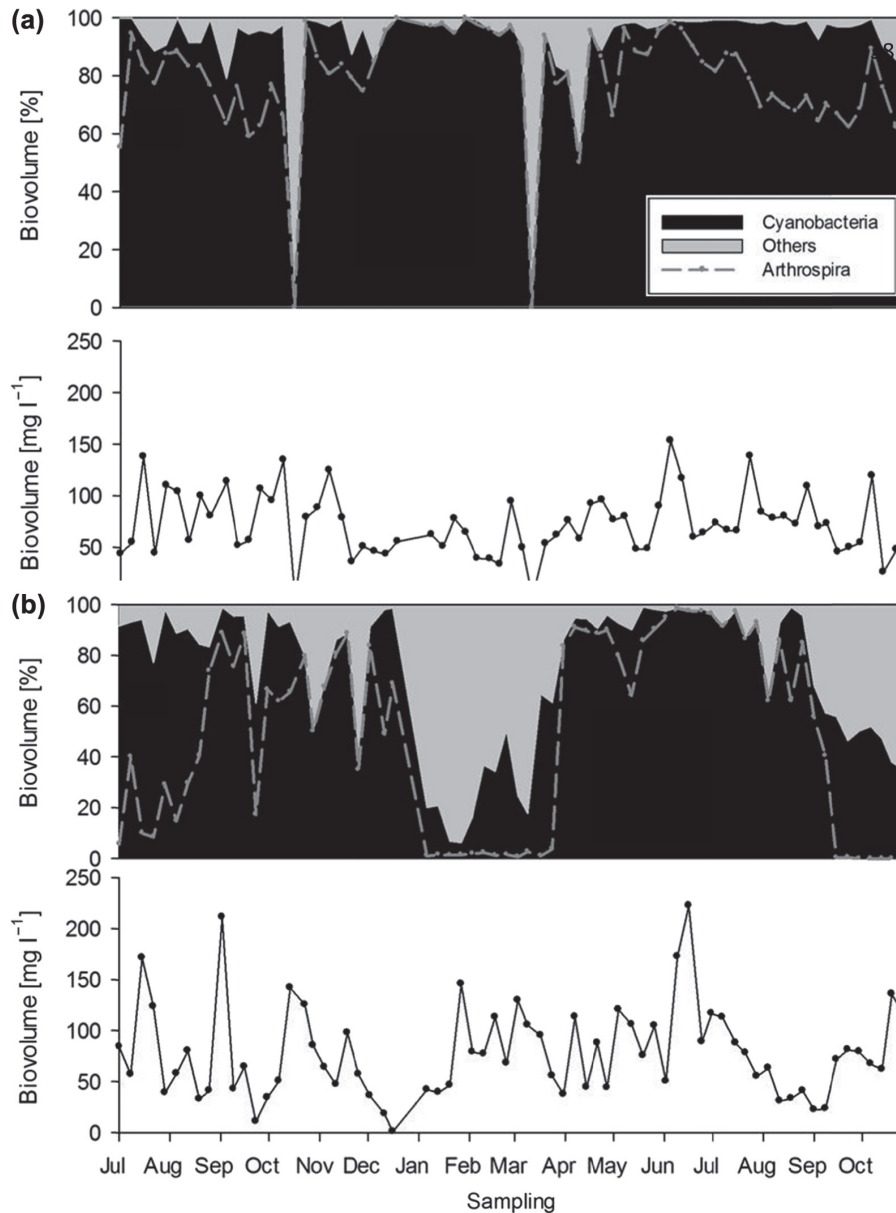
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Figure 2. Phytoplankton biovolume during the study showing *Arthrospira* (dashed line), total cyanobacteria (black area) and other algal groups (grey area) in percent (top) and absolute total biovolume (bottom). (2a) Bogoria, (2b) Nakuru; data modified from Kaggwa et al. (2013).

key factors in these die-offs. Food supply has been sufficient and in satisfying quality. At food shortage, flamingos usually start moving to other lakes (Tuite 1979, 2000, Kaggwa et al. 2013) indicating other reasons for die-offs.

Our study suggests excluding cyanobacterial toxins as well as major cause of mass die-off. We used different methods and equipment and never got any positive result. Potential toxin producers such as *Anabaenopsis* sp. were detected, but we did not observe *Microcystis*. Permanent pesticide and heavy metal discharging into the systems may also weaken the birds making them sensitive to infections. For lakes Bogoria, Nakuru and Elementaita, Ochieng et al. (2007) found heavy metal concentrations above the highest desirable level in drinking water (HDL; WHO) and most of the time above the maximum permissible level in drinking water (MPL; WHO). Krienitz and Kotut (2010) proposed

that consumed *Anabaenopsis* can clog the filters in the beaks due to its heavy mucilage. Clogging by aggregated *A. fusiformis* has already been described by Vareschi (1978), and we also observed such agglomerations in Bogoria, which could also contribute to a weakening of the flamingos. The current state of knowledge rather suggests that at certain times multiple causes including cyanobacteria aggregations weakens the bird population, making them vulnerable to bird diseases. As only flamingos are affected by this phenomenon, this points towards epizooty as cause of death. In fact, such diseases like avian tuberculosis have already been noted in these ecosystems (Kock et al. 1999).

There is still an urgent need for further comprehensive and systematic investigations, which hopefully will be funded even though flamingo die-offs occur erratically and last only a few days. Key problems seem to be the unpredictability of

flamingo mass mortality and its rapid development. This asks for 'stand by' experts, who must become active within hours to days. Because funding agencies will probably not support such projects based on many coincidences, environmental protection agencies are required. Cooperation between institutions offering state-of-the-art methods and local scientists would achieve the best results. Especially veterinarians should be included in this research topic to gain new insights into behavior of the birds and for diagnosis of avian diseases.

References

An, J. and Carmichael, W. W. 1994. Use of a colorimetric protein phosphatase inhibition assay and enzyme linked immunosorbent assay for the study of microcystins and nodularins. – Toxicon 32: 1495–1507.

Ballot, A. et al. 2004a. Cyanobacteria and cyanobacterial toxins in three alkaline rift valley lakes of Kenya – Lakes Bogoria, Nakuru and Elmenteita. – J. Plankton Res. 26: 925–935.

Ballot, A. et al. 2004b. Phylogenetic relationship of *Arthrospira*, *Phormidium* and *Spirulina* strains from Kenyan and Indian waterbodies. – Algological Studies 113: 37–56.

Belay, A. et al. 1993. Current knowledge on potential health benefits of *Spirulina*. – J. Appl. Phycol. 5: 235–241.

Capelli, B. and Cysewski, G. 2010. Potential health benefits of spirulina microalgae. – Nutrafoods 9: 19–26.

Dadheech, P. K. et al. 2009. Molecular detection of uncultured cyanobacteria and aminotransferase domains for cyanotoxin production in sediments of different Kenyan lakes. – FEMS Microbiol. Ecol. 68: 340–350.

Githaiga, J. M. 2003. Ecological factors determining utilization patterns and inter-lake movements of the flamingos in Kenyan alkaline lakes. – Nairobi, p. 228.

Greichus, Y. A. et al. 1978. Insecticides, polychlorinated biphenyls and metals in African lake ecosystems. III. Lake Nakuru, Kenya. – Bull. Environ. Contam. Toxicol. 19: 454–461.

Harper, D. M. et al. 2003. Aquatic biodiversity and saline lakes: Lake Bogoria National Reserve, Kenya. – Hydrobiologia 500: 259–276.

Hickley, P. et al. 2003. Bathymetry of lake Bogoria, Kenya. – J. E. Afr. Nat. Hist. 92: 107–117.

Kaggwa, M. N. et al. 2013. A detailed time series assessment of the diet of lesser flamingos: further explanation for itinerant behaviour. – Hydrobiologia 710: 83–93.

Kairu, J. K. 1996. Heavy metal residues in birds of Lake Nakuru, Kenya. – Afr. J. Ecol. 34: 397–400.

Kock, N. D. et al. 1999. *Mycobacterium avium* – related epizootic in free ranging lesser flamingos in Kenya. – J. Wildlife Diseases 35: 297–300.

Koenig, R. 2006. The pink death: die-offs of the lesser flamingo raise concern. – Science 313: 1724–1725.

Kotut, K. and Krienitz, L. 2011. Does the potentially toxic cyanobacterium *Microcystis* exist in the soda lakes of east Africa? – Hydrobiologia 664: 219–225.

Krienitz, L. and Kotut, K. 2010. Fluctuating algal food populations and the occurrence of lesser flamingos (*Phoeniconaias minor*) in three Kenyan rift valley lakes. – J. Phycol. 46: 1088–1096.

Krienitz, L. et al. 2003. Contribution of hot spring cyanobacteria to the mysterious deaths of lesser flamingos at Lake Bogoria, Kenya. – FEMS Microbiol. Ecol. 43: 141–148.

Krienitz, L. et al. 2005. Contribution of toxic cyanobacteria to massive deaths of lesser flamingos at saline-alkaline lakes of Kenya. – Verhand. Int. Verein Limnol. 29: 783–786.

Kurmayer, R. et al. 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. – Appl. Environ. Microbiol. 69: 787–795.

Lugomela, C. et al. 2006. Cyanobacteria blooms – a possible cause of mass mortality of lesser flamingos in Lake Manyara and Lake Big Momela, Tanzania. – Harmful Algae 5: 534–541.

McCall, J. 2010. Lake Bogoria, Kenya: hot and warm springs, geysers and Holocene stromatolites. – Earth-Sci. Rev. 103: 71–79.

Metcalf, J. S. et al. 2006. Analysis of the cyanotoxins anatoxin-a and microcystins in lesser flamingo feathers. – Toxicol. Environ. Chem. 88: 159–167.

Metcalf, J. S. et al. 2013. Amino acid neurotoxins in feathers of the lesser flamingo, *Phoeniconaias minor*. – Chemosphere 90: 835–839.

Motelin, G. et al. 2000. An ecotoxicological study of the potential roles of metals, pesticides and algal toxins on the 1993/5 lesser flamingo mass die-offs in Lake Bogoria and Nakuru, Kenya; and the health status of the same species of birds in the Rift Valley Lakes during the 1990s. – In: Proc. E. Afr. Environ. Forum, Nairobi, May 11–12, 2000.

Mussagy, A. et al. 2006. An experimental study of toxin production in *Arthrospira fusiformis* (Cyanophyceae) isolated from African waters. – Toxicon 48: 1027–1034.

Nasirwa, O. 2000. Conservation status of flamingos in Kenya. – Waterbirds 23: 47–51.

Ndetei, R. and Muhandiki, V. S. 2005. Mortalities of lesser flamingos in Kenyan Rift Valley saline lakes and the implications for sustainable management of the lakes. – Lakes Reservoirs Res. Manage. 10: 51–58.

Ochieng, E. Z. et al. 2007. Analysis of heavy metals in water and surface sediment in Five Rift Valley Lakes in Kenya for assessment of recent increase in anthropogenic activities. – Bull. Environ. Contam. Toxicol. 79: 570–576.

Oduor, S. O. and Schagerl, M. 2007. Phytoplankton primary productivity characteristics in response to photosynthetically active radiation in three Kenyan Rift Valley saline–alkaline lakes. – J. Plankton Res. 29: 1041–1050.

Sileo, L. et al. 1979. Microbacteriosis in the lesser flamingo of Lake Nakuru, Kenya. – J. Wildlife Diseases 15: 387–390.

Stewart, I. et al. 2008. Cyanobacterial poisoning in livestock, wild mammals and birds – an overview. – Adv. Exp. Med. Biol. 619: 613–637.

Tuite, C. H. 1979. Population size, distribution and biomass density of the lesser flamingo in the Eastern Rift Valley, 1974–76. – J. Appl. Ecol. 16: 765–775.

Tuite, C. H. 2000. The distribution and density of lesser flamingos in East Africa in relation to food availability and productivity. – Waterbirds Int. J. Waterbird Biol. 23: 52–63.

Vareschi, E. 1978. The ecology of Lake Nakuru (Kenya) I. Abundance and feeding of the lesser flamingo. – Oecologia 32: 11–35.

Vareschi, E. and Jacobs, J. 1984. The ecology of Lake Nakuru (Kenya). V. Production and consumption of consumer organisms. – Oecologia 61: 83–98.

Zaccara, S. et al. 2011. Lesser flamingo *Phoeniconaias minor* as a nomadic species in African shallow alkaline lakes and pans: genetic structure and future perspectives. – Ostrich 82: 95–100.