Characteristics of the phytoplankton community and bioaccumulation of heavy metals during algal blooms in Xiangjiang River (Hunan, China)

LI Jie¹, PENG FuLi², DING DongBo¹, ZHANG ShuBing¹, LI DeLiang³ & ZHANG Ting^{3*}

¹School of Biological Science and Technology, Central South University, Changsha 410013, China;

²Department of Ecology, China National Environmental Monitoring Centre, Beijing 100012, China;

³College of Animal Science and Technology, Hunan Agriculture University, Changsha 410128, China

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The frequency of algal blooms has increased in the mid and downstream reaches of the Xiangjiang River (Hunan, China), one of the most heavily polluted rivers in China. We identified the bloom-forming species in a bloom that occurred mid-late September 2010. In addition, we determined the extent of metal bioaccumulation in the algae and measured the toxicity of the algae using a mouse bioassay. Water samples were collected at upstream (Yongzhou), midstream (Hengyang), and downstream (Zhuzhou, Xiangtan, and Changsha) sites. The dominant species was *Aulacoseira granulata*, formerly known as *Melosira granulata*. The heaviest bloom occurred at Xiangtan and Changsha, where the number of *A. granulata* peaked at 1.3×10^5 filaments L^{-1} and chlorophyll *a* at $0.04 \text{ mg } L^{-1}$. Concentrations of Al, Fe, and Mn were 4.4×10^3 , 768.4, and 138.7 mg kg^{-1} dry weight in the phytoplankton. The bioaccumulation factor was 4.0×10^5 , 7.7×10^5 , and 3.2×10^3 , respectively. The heavy metal Pb had the greatest tendency to bioaccumulate among the highly toxic heavy metals, with a concentration of 19.2 mg kg^{-1} dry weight and bioaccumulation factor of 9.6×10^3 . The mouse bioassay suggested the bloom was toxic. The LD_{50} was 384 mg kg^{-1} and all surviving mice lost weight during the first 72 h after exposure. Our results demonstrate that blooms of *A. granulata* in rivers contaminated with heavy metals pose a threat to freshwater ecosystems and human health. Thus, measures should be taken to control eutrophication and heavy metal pollution in such rivers.

***Aulacoseira granulata*, bioaccumulation, diatom bloom, heavy metal, Xiangjiang River**

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Occurrences of algal blooms have increased in frequency in the last decade and have become a significant problem throughout the world. In China, heavy blooms have been documented in a number of lakes, including Taihu Lake (2340 km^2 , the third largest freshwater lake), Chaohu Lake (769.55 km^2), and Dianchi Lake (309 km^2) [1–3], and several rivers, including the Hanjiang River, a major tributary of the Yangtze River [4,5]. In addition, these blooms persist over an extended period. For example, a diatom bloom per-

sisted for approximately one month during 2010 in the Hanjiang River. Similarly, cyanobacterial blooms in Dianchi Lake often persisted throughout the year [2]. These long lasting algal blooms affect the appearance and quality of the water, thus decreasing the value of the water body for recreation, fishing, hunting, and aesthetic enjoyment. Furthermore, the blooms pose a risk for animal and human health and aquatic-ecosystem sustainability [3,6,7].

Algal blooms may be toxic to aquatic animals and humans. A small percentage of algal species (>100 currently identified) produce toxin chemicals, including 60 species of

*Corresponding author (email: tingzh0517@163.com)

Cyanobacteria, 30 species of Dinophyta, and a few species of the genus Bacillariophyta, Chrysophyta, Rhodophyta, Phaeophyta, and Chlorophyta [8]. Contamination of the water used for kidney dialysis with microcystin resulted in 100 of 131 patients developing acute liver failure, leading to 52 deaths in Brazil in 1996 [9]. Given the health risk, it is critical to determine whether the dominant species in a bloom produces toxins. In addition, algae are able to bioaccumulate and bioconcentrate other toxic pollutants. Algal toxins and other toxic pollutants may be transferred to species of a higher trophic level, including human beings, and threaten the health of these animals [10].

Xiangjiang River is one of the primary tributaries of the Yangtze River. It has a total length of 856 km, of which 670 km is within Hunan Province. The river flows from south to north through the following five administrative regions (in order from upstream): Yongzhou, Hengyang, Zhuzhou, Xiangtan, and Changsha. These regions constitute the center for politics, economics and culture, and are the most developed areas in Hunan Province [11,12]. Recently, algal blooms have occurred in the river throughout this region, particularly in mid-late October 2008 and late September 2010. The later bloom blocked the filters at the water treatment facility, causing a significant decrease in the supply of water to Changsha city due to decreased hydraulic pressure and flux. In addition, the water had an unappealing odor and posed a health risk. Despite the risk to human health, little is known about the bloom-forming species and phytoplankton community structure for these blooms. Furthermore, Xiangjiang River is subjected to heavy metal pollution. The bioaccumulation of heavy metals in phytoplankton increases the ecotoxicological risk to other aquatic organisms and local inhabitants. In the present study, therefore, our objectives were to (i) identify the bloom-forming species and phytoplankton community structure, (ii) measure the bioaccumulation of heavy metals in the phytoplankton, and (iii) assess the toxicity of the algal bloom and determine whether it poses a threat to freshwater ecosystems or human health.

1 Materials and methods

1.1 Water sampling

Water samples were collected at five sites on September 21 and 23, 2010, during the peak period of algal blooms. The sites were located upstream at Quhe Bridge in Yongzhou, midstream at Xiangjiang Gongtie Bridge in Hengyang, and downstream at Xiangjiang First Bridge in Zhuzhou, Xiangtan First Bridge in Xiangtan, and Yinpengling Bridge in Changsha (Figure 1). The samples were collected 0.5 m below the water surface and at least 5 m away from the riverbank using a stainless steel sampler. At each site we collected samples from three parallel sites that were perpendicular to both bank sides. The three samples were then mixed and placed in plastic bottles. We used 1 L of the

sample for quantitative analysis of phytoplankton and the remaining water was used for quantifying levels of chlorophyll *a* and metals. Samples of phytoplankton for qualitative identification were collected at the water surface using a 25 μ m phytoplankton net. Lugol's solution (final concentration 1%) was added immediately to the phytoplankton samples.

1.2 Phytoplankton identification and biodiversity index calculation

The phytoplanktons were identified to the species level under a light microscope (Motic BA400, China) following the descriptions in *The Freshwater Algae of China: Systematics, Taxonomy, and Ecology* [13] and Chen and Li [14]. The samples were then digested by acid for accurate identification of the dominant species as follows. Two milliliters of sample was washed and centrifuged (2000 $\times$ g, 5 min). We then added 0.5 mL concentrated sulfuric acid and five drops of nitric acid to digest the sample until the solution became transparent. After washing five times with distilled water by centrifugation (2000 $\times$ g, 5 min), the residues were subject to scanning electron microscopy (HITACHI S-570, Japan).

We quantified biodiversity using the Shannon-Wiener index [15,16]. Values of 0–1 indicate heavy pollution and 1–3 moderate pollution (within which 1–2 indicates α -pollution and 2–3 β -pollution). Values >3 indicate light or no pollution.

1.3 Phytoplankton counts and chlorophyll *a* determination

The quantitative analysis of phytoplankton was conducted 24 h after collection. We gently mixed 50 mL of the settled sample and transferred 100 μ L to a slide. The number of the dominant species was counted under a standard light microscope (Motic BA400, China). Filaments with at least three cells were counted.

The concentration of chlorophyll *a* was measured spectrophotometrically following extraction in 90% acetone by a freeze-thaw method according to the description in *Methods for Water and Wastewater Analysis* (4th edition) [17]. The sample was obtained by filtering at least 300 mL water through a 1.2 μ m Waterman GF-C membrane.

1.4 Heavy metal analysis

Both total and dissolved concentrations of metals in the water samples were measured. A 20 mL aliquot of water was filtered through a 0.45 μ m cellulose nitrate membrane for dissolved concentrations determination. All samples were collected in a polypropylene vial and acidified to pH<2 with suprapur nitric acid (1%). As, Cd, Cr, Pb, Al, Fe, and Mn were measured using inductively coupled plasma-atomic emission spectrometry (ICP-AES, Intrepid II

Figure 1 Study area and sites description in Xiangjiang River.

XSP, USA). This analysis was performed by the Analysis and Measurement Center, Central South University (Changsha, China). The detection limit for each of the metals was 3.0, 0.1, 0.4, 2.0, 1.2, 0.3, and 0.03 $\mu\text{g L}^{-1}$, respectively. The Chinese standards for drinking water quality (GB 5749-2006) were referenced to assess whether the element reached levels that posed a risk to human health [18].

1.5 Bioaccumulation

To measure the bioaccumulation of heavy metals samples of phytoplankton collected at Changsha were used. The sample was settled and washed three times. Observation under a light microscope suggested that there was no sand or other suspended particle material in the water sample. The phytoplankton in the samples consisted primarily (95%) of the dominant species. A 1 g aliquot of the sample was filtered through a pre-dried and pre-weighed 1.2 μm GF-C membrane, which was then dried in an oven at 80°C for at least 48 h to obtain a constant weight for the dried samples. A second 1 g aliquot of the fresh sample was then processed by suprapur nitric acid digestion and filtered with the GF-C membrane before ICP-AES analysis. Bioaccumulation fac-

tors were calculated by dividing metal (As, Cd, Cr, Pb, Al, Fe, and Mn) concentrations in the phytoplankton (mg kg^{-1} dry weight) by the dissolved concentrations of each metal in the water sample (mg kg^{-1}). For samples in which the dissolved concentrations were lower than the limits of detection, the lower detection limits were used to represent the concentration of heavy metals.

1.6 Mouse bioassay

Mouse bioassay was performed following the methods in the acute toxicity test standard GB 15193.3-2003 [19] and the manual on harmful marine microalgae (second revised edition) [20]. Healthy male Kunming mice (weight 23–26 g) were obtained from Changsha Dongchuang Animal Science Service Department. A 26.4 g of fresh sample collected at the Changsha site was diluted to 30 mL with 0.9% NaCl. The diluted sample was then freeze-thawed six times and centrifuged ($8228\times g$, 3 min). The toxicity of both the supernatant and the unextracted sample was tested. Dilutions of the supernatant and the unextracted sample were prepared over the following range in 0.9% NaCl: 1, 1/2, 1/4, 1/8, 1/16. The control group received 0.9% NaCl. An ali-

quot of 0.9 mL was injected into three mice for each solution and we documented mortality and measured weight during the 72 h after injection.

1.7 Statistical analysis

To evaluate the distribution and bioaccumulation of metals in phytoplankton, differences between samples before and after filtration were analyzed using a paired *t*-test. Spearman correlation was conducted to determine the relationship between total metal concentrations and chlorophyll *a*. All the statistical analyses were performed with SPSS (Version 14.0, USA). A *P*-value of ≤ 0.05 was considered to be significant.

2 Results

2.1 Dominant species, phytoplankton composition and biomass

The dominant species were long-chained cylindrical frustules, with golden-brown plastids arranged close to the periphery (Figure 2A). The terminal cell had 1–4 long spines around the margin of the valve (Figure 2A and B). Circular valves were about 20 μm in diameter, flat without areolae (Figure 2C). The mantle had a length of 15–20 μm , with 6–8 areolae per 10 μm . The areolae on the terminal cell were much coarser than those on other cells (Figure 2B). The short, linking spine had a triangular shape, terminating at the end of each costa (Figure 2D). Based on these characteristics, the species was identified as the centric diatom *Aulacoseira granulata*, formerly known as *Melosira granulata*.

A. granulata was most abundant (1.3×10^5 filaments L^{-1}) in Changsha and Xiangtan (Figure 3A). The concentration

of chlorophyll *a* peaked at 0.04 and 0.027 mg L^{-1} , respectively, in these regions. In Zhuzhou, the maximum density of *A. granulata* was 0.5×10^5 filaments L^{-1} . Conversely, at the upstream (Yongzhou) and midstream (Hengyang) sites, the density of *A. granulata* was $< 1 \times 10^3$ filaments L^{-1} (Figure 3A) and chlorophyll *a* concentrations were 5 $\mu\text{g L}^{-1}$ (Figure 3B). Our results suggest that *A. granulata* bloomed in the downstream section of the river, primarily around Xiangtan and Changsha.

In Changsha, the species diversity of phytoplankton was low. The dominant species (*A. granulata*) accounted for more than 95% of the algal biomass. The remaining 5% consisted of three species of diatoms (*Fragilaria*, *Bacillaria*, *Gyrosigma*), six species of Chlorophyta, including one of *Ulothrix*, one of *Scenedesmus*, two of *Pediastrum*, and two of *Eudorina*, two species of Cyanobacteria, *Oscillatoria* and *Microcystis*, one species of Pyrrophyta, and one of Cryptophyta. The Shannon-Wiener index was 0.67, indicating that the pollution was heavy. The species composition in Zhuzhou and Xiangtan was similar to that in Changsha. Though *A. granulata* was the dominant species in Yongzhou and Hengyang, its biomass was not sufficient to form a bloom. In Yongzhou, in addition to the species observed downstream, we found five species of diatoms, one each of *Cymatopleura*, *Cymbella*, and *Amphora* and two species of *Gomphonema* as well as two species of Chlorophyta, including one each of *Oocystis* and *Oedogonium*. No cyanobacteria *Microcystis* was found. The species biodiversity was significantly higher at the upstream site. The Shannon-Wiener index was 2.47, indicating β -pollution, much lighter than the pollution in Changsha.

2.2 Distribution and bioaccumulation of metals in Xiangjiang River

Among the most toxic metals, the concentration of Pb was 0.035 mg L^{-1} in Zhuzhou. As levels exceeded 0.01 mg L^{-1} at all sites, except the upstream site at Yongzhou. The levels of these two metals exceeded the levels safe for drinking water based on the Chinese standard GB 5749-2006 (Table 1). Total concentrations of both Cr and Cd were below the drinking water criteria at all sites, and Cd levels were generally below the method detection limits (Table 1). Neither the concentrations before and after filtration nor the total concentrations among the up, mid, and downstream sites differed significantly ($P > 0.05$), with the exception of Pb.

In contrast to the highly toxic metals, the levels of Al, Fe, and Mn increased gradually in a downstream direction. In Zhuzhou and Changsha, the levels of all three metals were higher than the safe drinking water limits, particularly in Zhuzhou where the concentrations of Al, Fe, and Mn were 0.37, 0.538, and 0.122 mg L^{-1} , respectively. These levels were much higher than the safe levels of 0.2, 0.3, and 0.1

Figure 2 Photos of *A. granulata* under light microscopy (A), band (B), valve (C), and conjunction between cells (D) under electron microscopy.

Figure 3 Number of *A. granulata* cells (A) and chlorophyll *a* content (B) at different sites of Xiangjiang River.

Table 1 Metal concentrations in the surface water of Xiangjiang River before and after filtration with a 0.45 μm nitrocellulose membrane and the bioaccumulation of metals in phytoplankton (unit: mg L^{-1})^{a)}

Metal		As	Cd	Cr	Pb	Al	Fe	Mn
Levels risky to human health in drinking water		0.01	0.005	0.05	0.01	0.2	0.3	0.1
Detection limit of ICP-AES		0.003	0.0001	0.0004	0.002	0.0012	0.0003	0.00003
Yongzhou	Before	ND	0.001	0.008	0.006	0.069	0.054	0.032
	After	ND	ND	0.005	ND	0.012	0.001	0.002
Hengyang	Before	0.014	0.001	0.006	0.006	0.039	0.048	0.022
	After	0.014	ND	0.008	ND	0.008	ND	ND
Zhuzhou	Before	0.02	ND	0.01	0.035	0.372	0.538	0.122
	After	0.018	ND	0.006	ND	0.033	0.024	0.062
Xiangtan	Before	0.013	ND	0.005	ND	0.278	0.277	0.07
	After	ND	ND	0.007	ND	0.022	0.004	0.002
Changsha	Before	0.013	0.001	0.006	ND	0.326	0.36	0.153
	After	ND	ND	0.005	ND	0.011	0.001	0.044
Concentration in phytoplankton (mg kg^{-1} dry weight)		4.210	0.789	5	19.2	4.4×10^3	768.4	138.7
Bioaccumulation factor		1.4×10^3	7.9×10^3	1.0×10^3	9.6×10^3	4.0×10^5	7.7×10^5	3.2×10^3

a) ND, not detected.

mg L^{-1} for these three elements (Table 1). The total concentrations of the three elements were significantly higher than the dissolved concentrations (Al, $P=0.037$; Fe, $P=0.050$; Mn, $P=0.020$). Furthermore, the concentrations of these elements were correlated to the concentrations of chlorophyll *a* at all sites except Zhuzhou (correlation coefficient=0.949, $P=0.051$).

Phytoplankton tended to primarily accumulate Al and Fe. The content of these two metals was 4.4×10^3 and 768.4 mg kg^{-1} dry weight and the bioaccumulation factors were 4.0×10^5 and 7.7×10^5 , respectively (Table 1). Mn had a lower tendency to bioaccumulate in phytoplankton, with a content of 138.7 mg kg^{-1} and a bioaccumulation factor 3.2×10^3 . Among the highly toxic heavy metals, Pb was accumulated more often than Cd, Cr, and As. The concentration of Pb was 19.2 mg kg^{-1} dry weight and the bioaccumulation factor was 9.6×10^3 . As was accumulated at a lower rate, with a concentration of 4.2 mg kg^{-1} dry weight and a bioaccumulation factor of 1.4×10^3 .

2.3 Mouse bioassay

All the mice treated with supernatant were less energetic and lost weight in the first 24 h, except the mice injected with 1/16th dose of supernatant. All mice had recovered their weight by 72 h (Figure 4A). We did not observe any mortality in the mice that were injected with supernatant. In contrast, a number of mice that were injected with the unextracted sample died. All the mice injected with the undiluted solution or the 1/2 strength dilution died within 24 h. Furthermore, one of the mice that received the undiluted solution died in the first 6 h. Two mice in the 1/4 strength group and one in the 1/8th strength group died within 36 h. There was no mortality in the group given a 1/16th dilution. All the mice lost weight in the first 24 h and did not recover their initial weight by 72 h (Figure 4B). The LD_{50} value was 384 mg kg^{-1} with a standard error of log transformed value of 0.12 and a 95% confidence interval of between 228.5–646.3 mg kg^{-1} .

Figure 4 Effect of injecting the supernatant (A) and the unextracted sample (B) of phytoplankton on mouse weight.

We compared the differences in metal concentrations between the unextracted sample and the supernatant. The concentrations of Al, Fe, and Mn were 269.9, 46.7, and 8.4 mg L⁻¹ in the complete sample. These were 163, 53, and seven times higher, respectively, than those in the supernatant. In addition, the heavy metal Pb was 1.2 mg L⁻¹ in the complete sample but undetectable in the supernatant. There was no difference in the concentration of the other metals between the complete sample and the supernatant.

3 Discussion

Prior studies of the Xiangjiang river have often emphasized the problems of heavy metal pollution [12,21]. In our research, the concentrations of As, Pb, Al, Fe, and Mn in the water near Zhuzhou surpassed the levels safe for drinking water. However, heavy metal pollution has decreased gradually since 2002 [11], which is reflected in our observation that the levels of Cd, Cr, and Pb were close to the drinking water criteria at most sites. Xiangjiang River has become eutrophic, particularly in the downstream sections near Zhuzhou, Xiangtan, and Changsha, due to increasing wastewater and agricultural pollution [22]. Total phosphorus and ammonia-nitrogen are the two major factors controlling water quality in the Xiangjiang River [11]. *A. granulata* is a classical indicator of eutrophic waters. It is often the dominant species in eutrophic freshwater areas around the world [23–25]. In our study, the total abundance of *A. granulata* was 1.3×10⁵ filaments L⁻¹ at Xiangtan and Changsha, which is comparable to levels in other studies [24] and close to the level (3.15×10⁵ filaments L⁻¹) reported in the Pearl River in China [24]. Thus, our data suggest that the Xiangjiang River is experiencing rapid eutrophication.

Diatoms are one of the main primary producers in aquatic ecosystems. They are considered to be passive participants in energy transfer in the food webs, being heavily grazed by their predators and exhibiting little or no anti-grazing capacity [26]. A number of diatom species produce the potent neurotoxin domoic acid, including *Pseudo-nitzschia*, *Nitzschia*, and *Amphora* [10]. However, most diatoms are thought to be beneficial to the growth and survival of marine and freshwater organisms, and to the trans-

fer of organic material through the food chain to top consumers and important fisheries. Of note in this region, the diatom *Stephanodiscus*, responsible for blooms in Hanjiang River, was also reported to be non-toxic (LD₅₀>10 g kg⁻¹ for mice) [5,27]. Recently however, researchers have noted that copepods, the principal predators of diatoms, that feed on certain diatoms produce abnormal eggs that either fail to develop to hatching or hatch into malformed (i.e., teratogenic) nauplii that die soon afterwards [28,29]. The cytotoxic compounds responsible for these effects are short chain polyunsaturated aldehydes (PUAs) and other oxygenated fatty acid degradation products [29,30]. They are cleaved from fatty acid precursors by enzymes that activate within seconds after crushing of cells [29]. The known PUA producing species include many common species, such as *Melosira*, *Odontella*, *Phaeodactylum*, *Rhizosolenia*, *Skeletonema*, *Stephanopyxis*, and *Thalassiosira*. Studies on other sources of PUAs suggest that they induce cellular toxicity in the liver, kidney, stomach, and lung [31]. They react with DNA and thus there are increasing concerns for a potential link between aldehydes and development of cancer [32]. However, there have been no reports of aldehyde induced death. In the present study, we speculate that PUAs contributed to the loss of body weight in the first 24 h in the group injected with supernatant. The group injected with unextracted water samples had an LD₅₀ of 384 mg kg⁻¹, which is higher than the LD₅₀ for *Microcystis* (7.3–14.4 mg kg⁻¹) [33–35]. The time to death (6–48 h) was also longer than for mortality caused by microcystins and other cyanotoxins [36,37]. Given the significantly higher concentrations of Al, Fe, Mn, and Pb in the unextracted sample, we conclude that the bioaccumulation of metals in the phytoplankton contributed to the mortality of the mice.

Algae possess a range of unique structures and physiological characters which give them a high tolerance to the accumulation of metals [38]. They have functional groups in the cell wall and extracellular components to reduce metal bioavailability. Some peptides and proteins chelate and sequester metals. In addition, the metabolic processes compartmentalize metals into a vacuole [38]. Our data suggest that Al is the most readily bioaccumulated metal. We documented levels of 4.4×10³ mg kg⁻¹ dry weight, which was higher than Al levels (1.5×10³–2.0×10³ mg kg⁻¹) in phyto-

plankton in the non-polluted lake Rostherne Mere, UK, where *Aulacoseira* and other diatoms are co-dominate [39]. The differences may be due to the concentration in the water. Studies have shown a strong correlation between the concentration of metals in the water and in algae. For example, chlorophyta, *Dunaliella tertiolecta*, contained (979 ± 236) mg kg⁻¹ Al at water concentrations of 0.5 mg L⁻¹. This increased to (1505 ± 162) mg kg⁻¹ at water concentrations of 1.0 mg L⁻¹ [40]. Bioaccumulation of Pb is also of concern as Pb exhibits a high degree of toxicity to living organisms. Adverse effects of Pb exposure have been noted in the brain, blood, kidney, cardiovascular, and reproduction system [41,42]. In our research, Pb levels in phytoplankton peaked at 19.2 mg kg⁻¹ dry weight with a bioaccumulation factor 9.6×10^3 . However, the bioaccumulation factor may in fact be higher than what we measured as levels were almost undetectable in the water. Other studies have suggested that the bioaccumulation of Pb may be 2–4 times greater than for Al [40]. In Toulon Bay, France, the bioaccumulation of Pb by phytoplankton varied between seasons. The lowest value was 48, but peaked at 1.6×10^4 , which is 67% higher than the value observed in our study [43].

In conclusion, blooms of *A. granulata* in the Xiangjiang River bioaccumulate high levels of Al, Fe, and Pb. Inadvertently consuming these phytoplankton during a bloom represents a high risk to human and aquatic animal health. Given these risks, measures should be taken to control eutrophication and heavy metal pollution in this river.

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