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Corresponding Author: Dr. Frédéric Pitois, Ph.D.

Corresponding Author's Institution: Limnologie sarl

First Author: Frédéric Pitois, Ph.D.

Order of Authors: Frédéric Pitois, Ph.D.; Chantal Vezie, Ph.D.; Isabelle Thoraval, MSc; Estelle Baurès, Ph.D.

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**Improving Microcystin monitoring relevance in recreative waters: a regional case-study
(Brittany, Western France, Europe)**

Authors : Frédéric Pitois^{a,b,c}, Chantal Vezie^a, Isabelle Thoraval^a, Estelle Baurès^{b,c}

Authors affiliation :

a- Limnologie sarl, 16 rue Paul Langevin, 35200 Rennes, France

b- EHESP Rennes, Sorbonne Paris Cité, Avenue du Professeur Léon Bernard, CS 74312, 35043 Rennes
Cedex, France

c- Inserm, U 1085-IRSET, LERES, France

Authors mail adress :

Frédéric Pitois: fred.pitois@limnosphere.com

Chantal Vezie: chantal.vezie@limnosphere.com

Isabelle Thoraval: isabelle.thoraval@limnosphere.com

Estelle Baurès: estelle.baures@ehesp.fr

Corresponding author:

Frédéric Pitois, Limnologie sarl, 16 rue Paul Langevin, 35200 Rennes, France

fred.pitois@limnosphere.com

Abstract

Cyanobacteria and their toxins are known as a health hazard in recreative and distributed waters. Monitoring data from 2004 to 2011 were collected at regional scale to characterize exposition parameters to microcystins in Brittany (Western France). The data show that cyanobacteria populations are experiencing a composition shift leading to a longer duration of cell densities higher than WHO alert levels 2 and 3. Microcystins however appear to be more frequently detected with subacute concentrations in low cell density samples than in high cell density samples or during bloom episodes. Positive relations are described between microcystin concentrations, detection frequencies and cyanobacteria biovolumes, allowing for a novel definition of alert levels and decision framework following WHO recommendations.

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1- Introduction

Toxic cyanobacteria are known as a potential health hazard since the 1990s, and three major exposure routes are currently recognized: i) through dermal contact and accidental inhalation/ingestion during recreational activities in raw waters (Osborne et al., 2007; Stewart et al., 2006; Baker et al., 2008 and 2010); ii) through ingestion of drinking water produced from a contaminated resource (Byth, 1980; Griffiths and Saker, 2003; Merel et al., 2013) and iii) through the ingestion of cyanobacteria-based food ingredients, either directly as dietary compliments (Gilroy et al., 2000; Heussner et al., 2012; Rellan et al., 2009; Vichi et al., 2012) or indirectly via contaminated seafood, fishes or shellfishes (Ibelings and Chorus, 2007). Low-level chronic exposure through these routes is currently suspected of the highest human health impact (Spoof, 2005; Li et al., 2011, Wierich and Miller; 2014).

Water exposition routes are the most documented since the publication of WHO provisional guidelines for recreative and treated waters (Chorus and Bartram, 1999). Recreational water surveys involve a tiered management framework based on biomass proxies (chlorophyll a, cyanobacteria cell density), and the proposed decision scheme has been implemented by most countries where cyanobacteria are monitored (Ibelings et al., 2014). To summarize, this decision framework requires cyanobacteria to be quantified, and cell densities to be compared to Alert Level values: Alert Level 2 (20 000 cell/ml) implies water users information, whereas above Alert Level 3 (100 000 cell/ml) microcystin occurrence is likely and microcystin analysis is recommended. Microcystin concentrations are then compared with guideline values calculated according to a daily No Observed Adverse Effect Level (NOAEL) intake of 40 µg/kg bodyweight (Falconer et al., 1994; Codd et al., 2005). WHO guideline for recreational activity suspension is 20 µg/l microcystin LR, whereas French regulation, based on different intake hypotheses, adopted a chronic exposure guideline of 13 µg/l and an acute exposure guideline of 80 µg/l since 2013.

While proliferation episodes are widely documented and subjected to research work, chronic exposure, subacute toxin concentration and low-biomass effects are on the other hand less often

considered. They can however be seen as a health hazard for particular populations such as children, pregnant women, asthmatic or atopic people... (Li et al., 2011; Weirich and Miller, 2014). Cyanobacterial toxins are for example known to be associated with carcinogenesis (Zegura et al., 2011), cytotoxicity ((Young et al., 2008) and have been shown to act as endocrine disruptors (Sychrova et al, 2012) or allergenic/pyrogenic agents, either as aerosols (Kirkpatrick et al., 2011 ; Cheng et al., 2007) or through direct contact with raw waters (Pilotto et al., 2004; Kirkpatrick et al., 2010 and 2011; Ohkouchi et al., 2015).

Public health surveys of recreational inland waters in Brittany (north western France) are held since 2003, and monitoring data have shown subacute toxin concentrations to be the most frequently encountered. In this context the present exploitation of water quality data from 26 recreational lakes collected between 2004 and 2011 was aimed at answering two questions: first, does the current monitoring framework derived from the 1999 WHO recommendations accurately account for microcystin exposure in recreational waters? Secondly, if this is not the case how can it be improved in terms of exposure representation and cost efficiency?

2- Materials and methods

Our study is based on public health weekly survey data from 26 lakes used as recreational areas or water resources monitored every year from 2004 to 2011 in Brittany (north-western France, Figure 1). These sites were selected according to cyanobacteria survey continuity criteria. Only 26 sites out of a total of 40 could be considered as continuously monitored from June to September since 2004.

Figure 1

Cyanobacteria data, i.e. cell densities and microcystin concentrations, were collected from the regional public health authorities (French Agence Régionale de Santé of Brittany). All samples were

analyzed by four different local laboratories for cyanobacteria composition and microcystin concentration.

Cyanobacteria were identified and counted with light microscope according to Utermöhl (1958). All monitoring data were first examined as cell densities, to comply with WHO alert level thresholds (Chorus and Bartram, 1999), and then converted to cell biovolumes using mean cell dimensions obtained from cyanobacteria reference works (Geitler, 1932; Komarek and Anagnostidis, 1999 and 2005; Komarek, 2013) and relevant geometric formulas (US Environmental Protection Agency, 2010). This conversion was meant to account for differences in biomass contribution induced by larger taxa associated with lower cell densities (e.g. *Microcystis*, *Woronichinia*, etc.) vs. smaller taxa associated with larger cell densities (e.g. *Aphanocapsa*, *Aphanothece*, etc.).

All toxin analyses were conducted on the same samples used for species composition analysis. Toxin analyses were systematically performed on all samples reaching WHO alert level 3, but could be performed on lower cell density (e.g. WHO alert level 2). Sample preparation involved a preliminary cyanobacteria cell lysis step with methanol (Lawrence et al., 2001) to account for total (dissolved and intra-cell) toxin concentrations. Depending on the laboratories, Microcystins (MCs) were analyzed either with LC/MS, HPLC or ELISA immunoassays. These methods quantification limits ranged from 0.05 to 0.2 µg/L. All laboratory results were harmonized to a common quantification limit, i.e. 0.2 µg/L.

From 2004 to 2011 in Brittany, cyanobacteria were analyzed in 3 278 water samples; MCs analyses were conducted on a total of 719 samples (i.e. 23% of all samples) and MCs were detected in 159 samples (i.e. 22% of all samples analyzed for MCs).

Frequency distribution analyses were carried out on grouped data with classes of 1, 2 and $5 \cdot 10^i$ with $i = -1; 0; 1; 2$ for MCs concentrations, $i = 4; 5; 6$ for cell densities, and $i = 5; 6; 7$ for cell biovolumes.

The Monitoring Cost Index used from section 3.3 onwards was calculated in two steps. First the mean analytical cost at regional scale was calculated for the current situation, i.e. 23 % samples analyzed

for MCs. Then a new mean regional cost was estimated for any proportion of samples analyzed for MCs according to monitoring hypothesis.

Minimum biovolume thresholds were graphically derived from MCs concentration classes. These thresholds were defined as the minimal biovolume for a positive detection of MCs class (n-1). For MCs = 1 µg/l for example, the threshold biovolume was set as the minimum cell volume allowing an observation of $0.5 < \text{MCs} < 1 \text{ µg/l}$; for a target MCs of 20 µg/l, the threshold was the minimal biovolume giving $5 < \text{MCs} < 10 \text{ µg/l}$, etc...

3- Results and discussion

3.1- Cyanobacteria occurrences and biomass

All data together, maximal cell densities ranged every year from 1.9 to $6.5 \cdot 10^6$ cell/ml, with weekly average values from 10 600 to 452 000 cell/ml. Quantile distributions of cell densities and WHO alert thresholds have already been described in a previous work (Pitois et al., 2014a). To summarize, the mean weekly values tended to increase regularly from 2004 to 2011, in particular because of an increase of the lower, spring densities (Figure 2). Cell densities above WHO alert level 2 (20 000 cell/ml) accounted for 48% of all samples (from 41 to 57% results every year). WHO alert level 3 was exceeded in 23% of the data distribution, and in 22 to 31% of all samples. Maximal cell densities were usually observed in mid-summer, i.e. from July, 15th to August, 25th. Peak densities tended however to occur earlier (i.e. July, 15th-22nd) from 2006 to 2008, and later (i.e. August, 15th-25th) in 2004-2005 and from 2009 to 2011.

Figure 2

Maximum biovolumes ranged from 130 to $210 \cdot 10^6 \text{ µm}^3/\text{ml}$, with mean weekly values from 4 to $9.9 \cdot 10^6 \text{ µm}^3/\text{ml}$ (Figure 3). Compared to cell densities evolution, cell biovolumes appeared almost

constant: from one year to the other biovolume range covered 2 orders of magnitude whereas cell density variations could reach 4 to 5 orders.

Maximum cell biovolumes were usually observed in mid-summer (from July, 11th to August, 16th) except for 2004 (early September). Maximal biovolume and cell density values were usually encountered in the same week from 2006 to 2010 but peak biovolumes appeared 2 weeks earlier in 2004-2005 and 7 weeks later in 2011.

Figure 3

The parallel observation of a gradual cell density increase while cell biovolume remained fairly constant over the years illustrates a shift in cyanobacterial species composition (Figure 4), with smaller size taxa replacing larger size species from 2008 onward. This is emphasized in Figure 4, showing in parallel an increase of the combined total cell density smaller Chroococcales such as *Aphanothece sp.*, *Aphanocapsa sp.*, *Merismopedia sp.* ... and a decrease of the combined cell density for *Microcystis sp.* and *Anabaena sp.* (largest taxa observed in Brittany).

Figure 4

3.2- Microcystins concentrations and detection frequencies

MCs concentrations appeared lower than 1 µg/l in 70 % of all analyzed samples, whereas 8% were higher than 13 µg/l (chronic exposition guideline under current French regulation), 5% higher than 20 µg/l (WHO provisional guideline for recreative waters) and 2.5% higher than 80 µg/l (acute exposition guideline, current French regulation).

Annual data show that at regional scale 92% of monitoring weeks showed less than 50% of all samples with positive MCs detection (Figure 5), whereas in some cases (10 weeks in 8 years, i.e. 8% monitoring duration) more than 60% samples could show positive MCs detection.

Minimal detection frequencies were observed in 2004, 2008 and 2011 with 30 to 50% max. positive samples, whereas 2005, 2006, 2009 and 2010 could reach 100 % positive detections during one week every year.

Figure 5

Annual peak detection frequencies never appeared to be synchronized with max. cell densities, but could be observed from 5 weeks earlier to 7 weeks later without definite pattern. In the same way, max. detection frequencies were offset relative to max. cell biovolumes: they were observed 5 to 8 weeks before peak biovolume from 2004 to 2007, and 3 to 7 weeks after peak biovolume from 2008 to 2011.

An inverse relation was found between cell density and MCs detection frequency (Figure 6a), as max. MCs detection frequencies (32 % samples) appeared in the 50 to 100 000 cell/ml range. The lowest detection frequencies (12-15 % samples) were encountered for cell densities higher than 200 000 cell/ml. The maximal MCs concentrations, up to 234 µg/l, were observed in the highest cell density samples (higher than 500 000 cell/ml). The MCs concentrations encountered from 50 to 100 000 cell/ml, although lower, could however reach 16 to 22 µg/l. In such cases MCs can exceed WHO guidelines for recreative waters and pose a health hazard to children and young adults. On the other hand, a positive relation was found between cell biovolume and MCs detection frequency (Figure 6b), reaching 27 % positive MCs detections for $5 \cdot 10^6 \mu\text{m}^3/\text{ml}$, and a maximum of 31-33 % positive detections above $5 \cdot 10^7 \mu\text{m}^3/\text{ml}$. This relation paralleled another positive relation between cell biovolume and MCs max concentrations reaching 11 to 32 µg/l from $5 \cdot 10^6$ to $5 \cdot 10^7 \mu\text{m}^3/\text{ml}$, and 126 to 234 µg/l above $5 \cdot 10^7 \mu\text{m}^3/\text{ml}$.

Figure 6a, 6b

3.3- Alert level assessment

From 2004 to 2011 WHO alert level 2 (20 000 cell/ml) was exceeded in 49% of all samples and MCs were detected in 22% of all samples reaching this threshold. In the same time, WHO alert level 3 was exceeded in 24% samples and MCs were detected in 20% of corresponding samples.

The data for 2004-2011 show that MCs could be detected (i.e. $> 0.2 \mu\text{g/l}$) in samples with cell densities as low as 340 cell/ml. On the other hand, the minimal cell density for $1 \mu\text{g/l}$ MCs was 9 259 cell/ml (i.e. 62% samples), and 11 680 cell/ml (i.e. 65% samples) for $20 \mu\text{g/l}$ MCs. Searching for MCs when cell density reaches these values would result in a regional monitoring cost multiplied by 1.5 and 1.6 in comparison with analyses for cell densities higher than WHO alert 3.

These results show that MCs monitoring protocols based on high cell densities tend to underestimate toxin presence, questioning cell density relevance as a risk indicator. On the other hand, the positive relations underlined between cell biovolume, MCs detection frequency and MCs concentrations can be used for risk management in the full range of cyanobacteria concentration, from the lowest (i.e. the most numerous cases) to the highest cell densities.

According to the relations underlined between cell biovolume, MCs detections and MCs concentrations, the minimal biovolume necessary to observe target MCs concentrations (TMC) can be calculated (Figure 7), thus allowing for novel alert levels definition.

Figure 7

Alert levels corresponding to target MCs concentrations can be, in our case, graphically derived from figure 7, The cell biovolume thresholds corresponding to MCs concentrations of $1 \mu\text{g/l}$ and $20 \mu\text{g/l}$ were determined to be $455\,000 \mu\text{m}^3/\text{ml}$ and $4\,500\,000 \mu\text{m}^3/\text{ml}$ respectively.

Searching MCs with a TMC = $1 \mu\text{g/l}$ in samples with a biovolume higher than $450\,000 \mu\text{m}^3/\text{ml}$, i.e. 56% of all samples, would lead to 23% positive detections for a regional cost index of 1.4 compared to WHO alert level 3. Setting the TMC at $20 \mu\text{g/l}$ and $4\,500\,000 \mu\text{m}^3/\text{ml}$, i.e. 22% samples, would lead

to 26% positive detection with a regional cost index of 0.97, similar to the current monitoring cost based on WHO alert level 3.

Further comparisons between biovolume thresholds and low cell density samples show that:

- 30% of all samples had a cell biovolume higher than 450 000 $\mu\text{m}^3/\text{ml}$ and cell density lower than 100 000 cell/ml. Positive MCs detections occurred in 37% of these samples.
- 5% of all samples had a cell biovolume higher than 4 500 000 $\mu\text{m}^3/\text{ml}$ and cell density lower than 100 000 cell/ml. Positive MCs detections occurred in 51% of these samples.
- Conversely 0.8% of all samples had a cell density higher than 100 000 cell/ml and a biovolume lower than 450 000 $\mu\text{m}^3/\text{ml}$. None of them was positive for MCs.

4- Conclusion

In France as in most other countries where cyanobacteria are monitored, MCs monitoring in recreational waters is focused on proliferation episodes estimated through cell densities analysis, and MCs analysis is mandatorily required above WHO alert level 3, i.e. 100 000 cell/ml. As analyses are optional below WHO Alert Level 3, data on toxin occurrence for low cell densities are scarce and public health managers can question this threshold relevance with regard to microcystin exposure estimation. Chorus and Bartram (1999) insisted on the necessary local assessment and validation of WHO guidelines. Local adaptation can then lead to new decision frameworks, with the use of different alert levels based on cell densities or cell biovolume (Ibelings et al., 2014) or other biomass proxies (Watzin et al., 2006; Rogalus and Watzin, 2008).

In our view, a public health cyanobacteria-MCs monitoring protocol must be representative of all - including subacute- exposition situations. The simplest solution to achieve this would be systematic analysis of cyanobacteria and toxin concentrations. However, this is impossible because of cost issues. As the samples analyzed for toxin concentration must be selected, the selection process must conduct to the highest possible toxin detection frequency in all selected samples.

The present study allowed a comparison between cell density and cell biovolume as indicators of microcystin exposure in recreational waters. Our results show that a monitoring protocol based on cell density thresholds matches available observations from the literature related to acute MCs concentrations: the highest MCs concentrations are encountered in the highest cell density or biovolume samples. From this point of view, using biovolume as an indicator doesn't bring much supplementary information.

Using cell biovolume seems however adapted to the most numerous cases in Brittany, for cyanobacteria cell densities lower than WHO alert level 3, when risks are not considered by water users, i.e. no bloom, no green coloration... no 'hazard' feeling (Hunter et al., 2012). In these cases toxin detection frequencies are the highest and microcystin concentrations can be a health hazard for children and young adults. A MCs monitoring based on cell biovolumes seems better suited to account for toxin exposure than the classical cell density scheme.

Cell biovolume thresholds can be defined to account for target MCs concentrations (TMC) while maintaining analytical costs similar to the actual monitoring scheme at regional scale. Cost index may however vary from lake to lake, as higher cell densities and higher cell biovolumes are not always encountered in the same sites. In the present study 30% of all samples had high biovolumes and low cell densities (with TMC = 1 µg/l), meaning that part of the monitoring cost would be redistributed from higher densities/lower biovolume lakes to lower densities/higher biovolumes lakes.

The present results were obtained in an oceanic climate context, in mostly eutrophic lakes, with highly diverse planktonic populations. Cyanobacteria taxonomic composition and MCs production can be affected by various abiotic parameters such as meteorology (light, temperature: Wiedner et al., 2007; Pitois et al., 2014b; Rigosi et al., 2015), hydrology (Carvalho et al., 2011) or nutrient balance (Dolman et al., 2012), and even synergistic inter-species activity (Garcia Nieto et al., 2011). The method for cell biovolume alert levels should be validated on a larger geographical basis, as the proposed biovolume thresholds will not necessarily be relevant in other climatic or trophic conditions.

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Figure captions

Figure 1: Spatial distribution of the 26 investigated sites in Brittany (France).

Figure 2: Mean weekly cell densities from June 2004 to September 2011.

Figure 3: Mean weekly cell biovolumes from June 2004 to September 2011.

Figure 4: Cyanobacteria generic composition evolution from June 2004 to September 2011.

Figure 5: MCs and weekly detection frequencies from June 2004 to September 2011.

Figure 6: Microcystin detection frequencies vs. cell densities (a) and cell biovolume (b). Only class means are illustrated for better clarity.

Figure 7: Minimal biovolume thresholds relation with target MCs concentrations.

Manuscript highlights

- Toxic cyanobacteria data were collected from an 8-years regional public health survey
- Microcystin exposure was estimated in the raw waters from 26 lakes
- Subacute toxin concentrations more frequently encountered than acute concentrations
- Alert levels based on cell densities and cell biovolumes are compared in view of a better exposure representativity

Figure 1

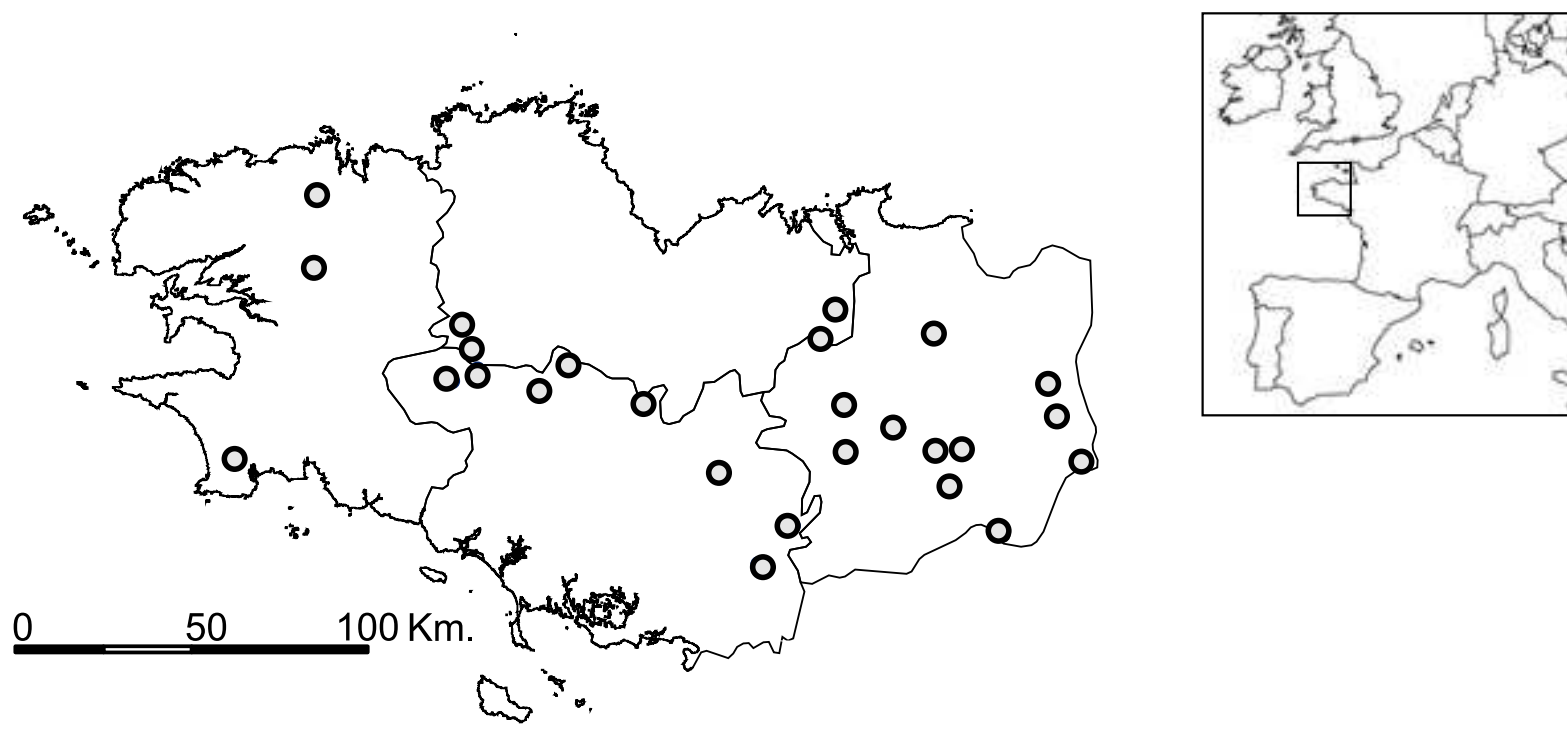


Figure 2

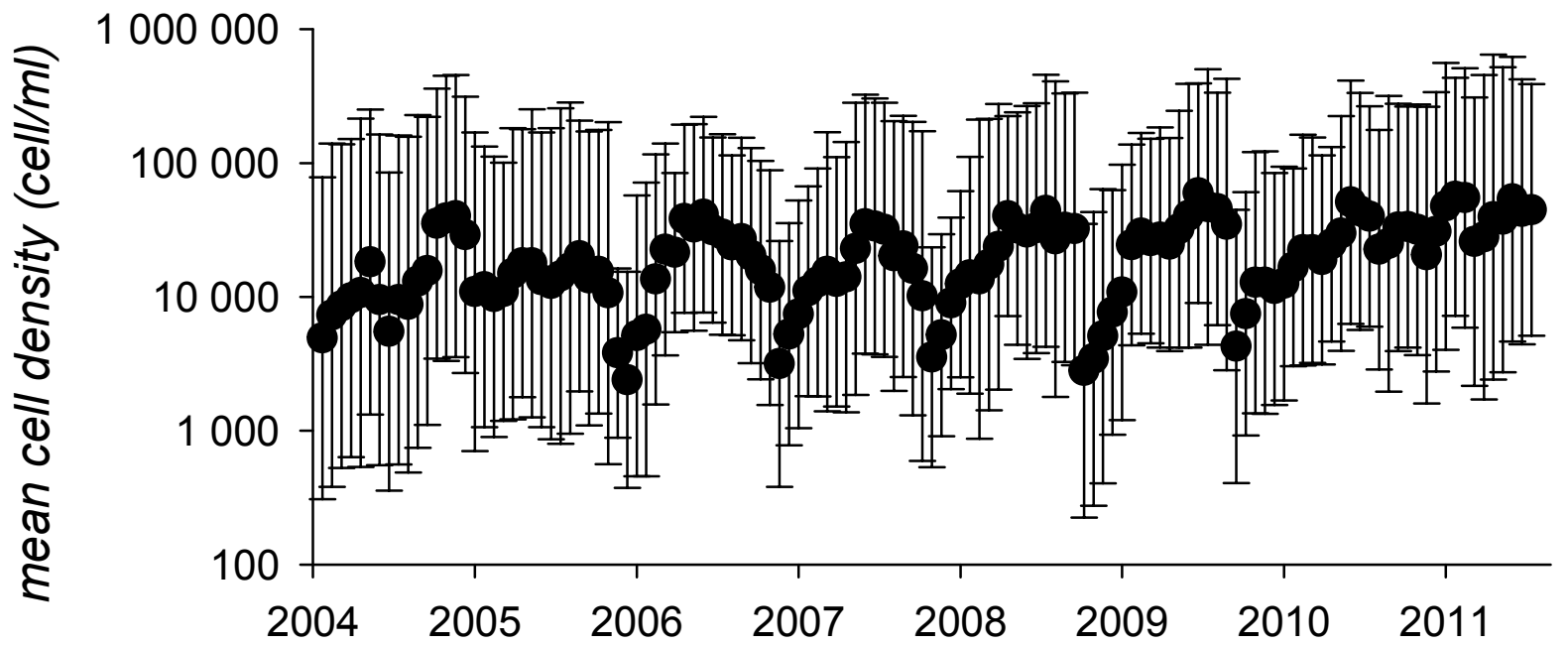


Figure 3

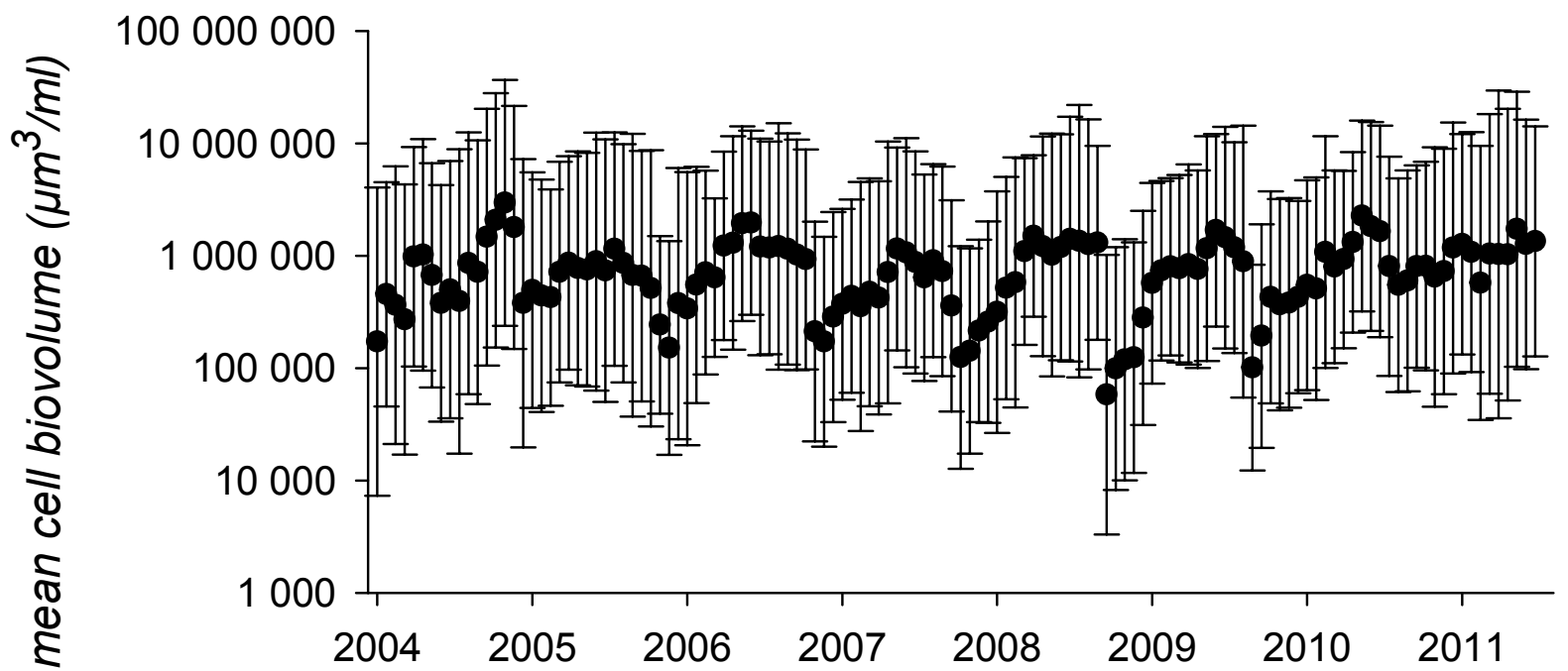


Figure 4

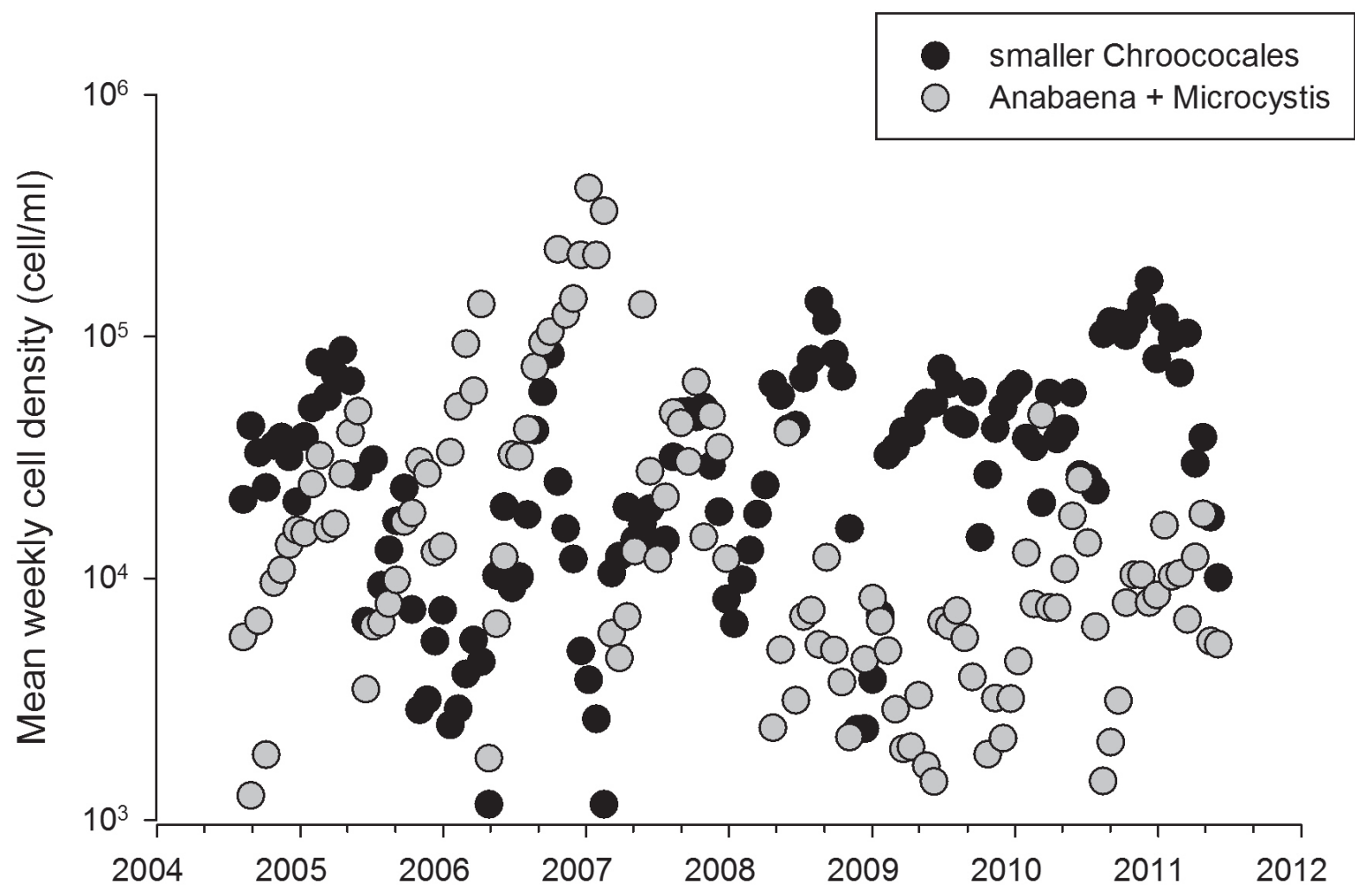


Figure 6a

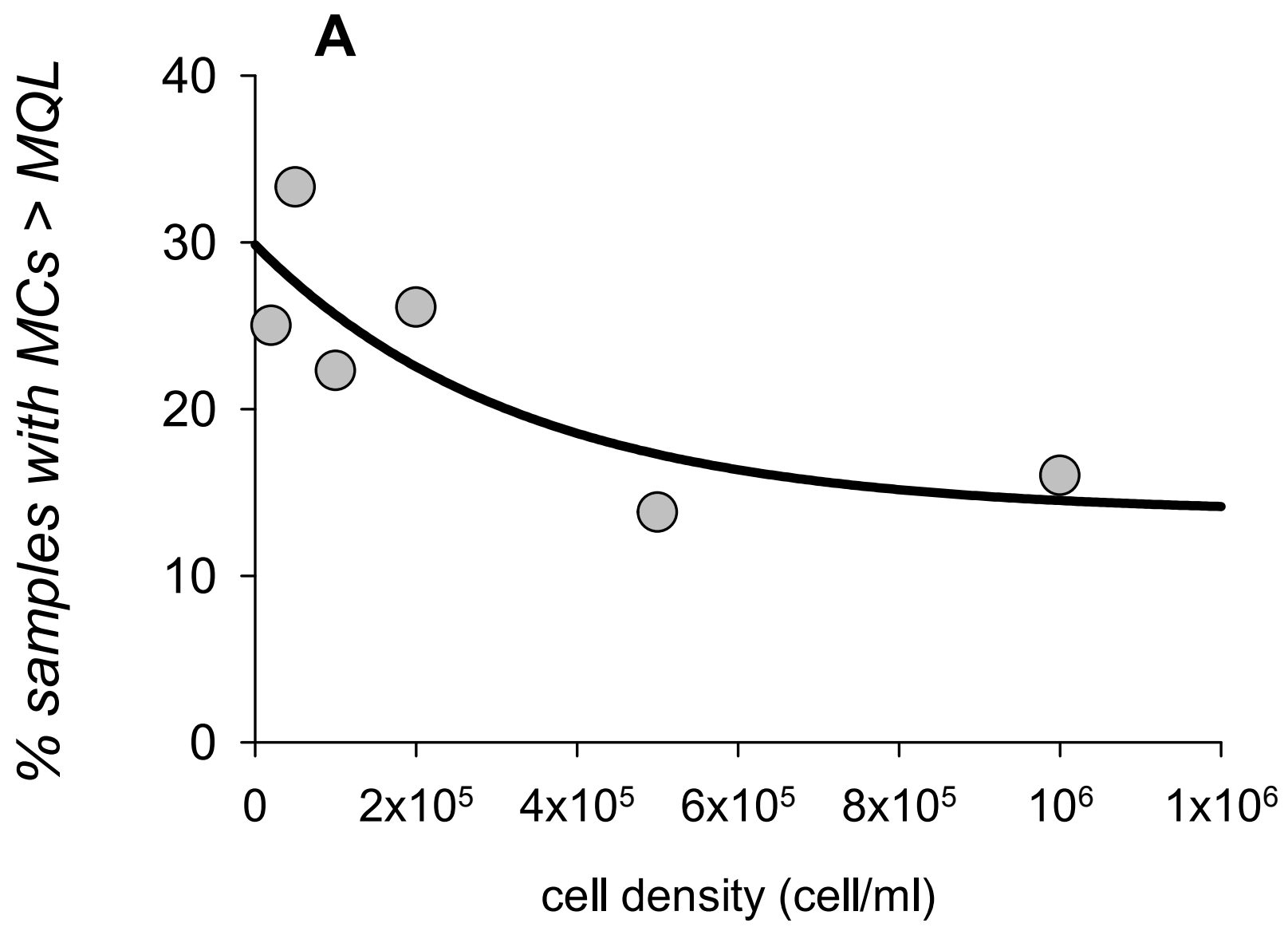


Figure 6b

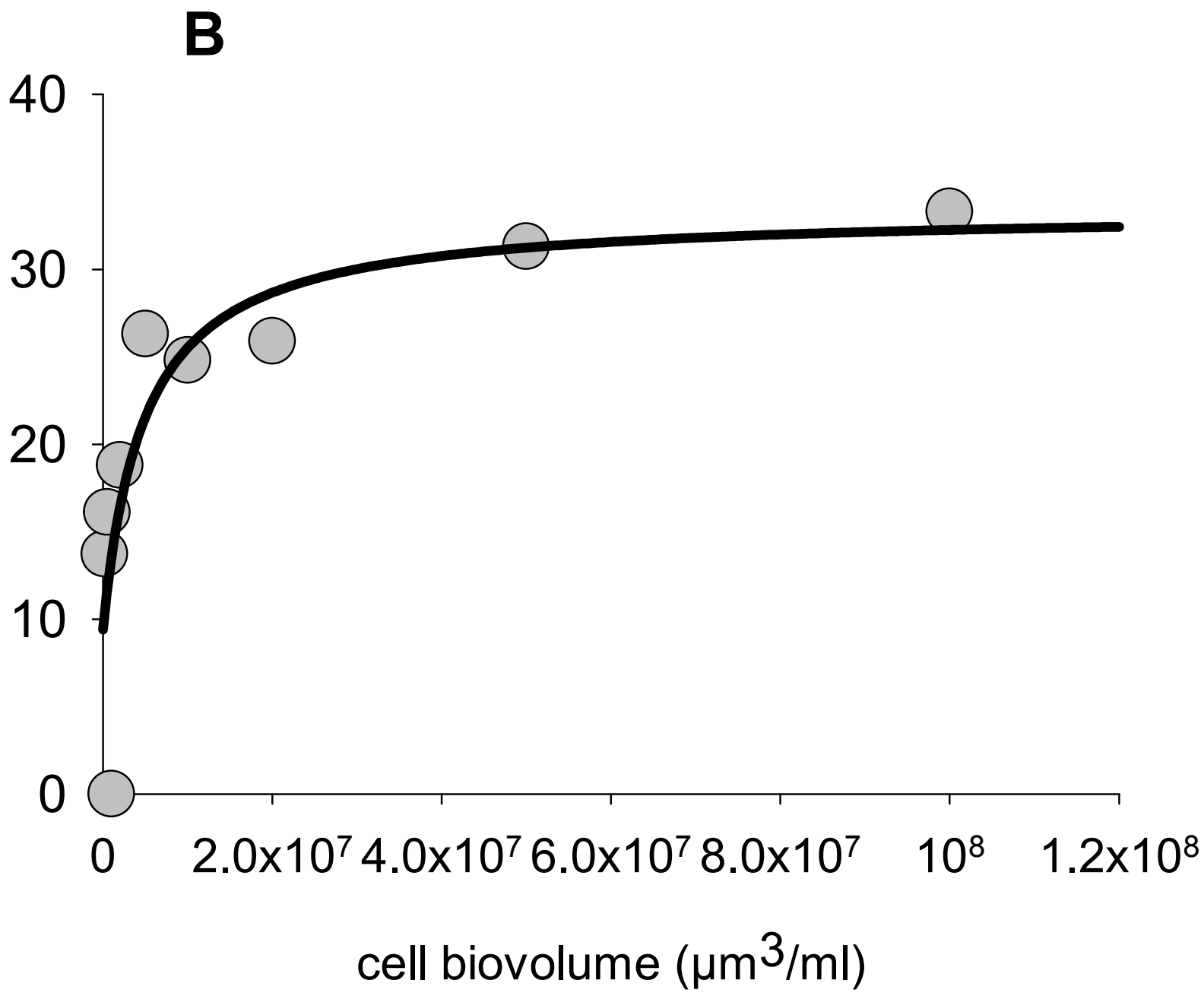


Figure 7

