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Abstract

Sampling cyanobacteria in freshwater ecosystems is a crucial aspect of monitoring programs in both basic and applied research. Despite this, few papers have dealt with this aspect, and a high proportion of cyanobacteria monitoring programs are still based on monthly or twice-monthly water sampling, usually performed at a single location. In this study, we conducted high frequency spatial and temporal water sampling in a small eutrophic shallow lake that experiences cyanobacterial blooms every year. We demonstrate that the spatial and temporal aspects of the sampling strategy had a considerable impact on the findings of cyanobacteria monitoring in this lake. In particular, two peaks of *Aphanizomenon flos-aquae* cell abundances were usually not picked up by the various temporal sampling strategies tested. In contrast, sampling once a month was sufficient to provide a good overall estimation of the population dynamics of *Microcystis aeruginosa*. The spatial frequency of sampling was also important, and the choice in the location of the sampling points around the lake was very important if only two or three sampling points were used. When four or five sampling points were used, this reduced the impact of the choice of the location of the sampling points, and allowed to obtain fairly similar results than when six sampling points were used. These findings demonstrate the importance of the sampling strategy in cyanobacteria monitoring, and the fact that it is impossible to propose a single universal sampling strategy that is appropriate for all freshwater ecosystems and also for all cyanobacteria.

Keywords: sampling strategy, cyanobacteria, spatiotemporal dynamic, *Microcystis aeruginosa*, *Aphanizomenon flos-aquae*

1 Introduction

Due to eutrophication and, to a lesser extent, to climatic changes (Markensten et al., 2010; Paerl and Huisman, 2009) cyanobacterial blooms seem to be increasing in freshwater ecosystems worldwide. These blooms severely disrupt the functioning of these ecosystems and potential water use. Furthermore, many cyanobacterial species are able to produce a variety of toxic metabolites, which can be harmful to both human (Kuiper-Goodman et al., 1999) and animal (Codd et al., 2005) health. For these reasons, numerous attempts have been made in the last 20 years to elucidate the factors that control cyanobacterial blooms and toxin production, and thus to make it possible to evaluate better the health risks associated with bloom events. From all these studies, it is clear that the spatial distribution of cyanobacteria in freshwater ecosystems can display marked horizontal and vertical variations (Porat et al., 2001; Welker et al., 2003). Moreover, by means of a real time PCR analysis of a gene involved in the biosynthesis of microcystins we have shown that considerable fluctuations can also occur in the proportions of potentially microcystin-producing and non-producing cells during the course of *Microcystis aeruginosa* blooms (Briand et al., 2009). Similar results have been found for various different *M. aeruginosa* populations located in the same geographic area (Sabart et al., 2009), which makes it difficult to manage the health risks associated with these events.

All these studies indicate that the sampling strategy used for monitoring cyanobacteria is a critical aspect, both in basic research on cyanobacteria, (e.g. investigation of the factors and processes involved in the development of the blooms), and in applied research, (e.g. implementing monitoring programs of these microorganisms in freshwater ecosystems used to provide drinking water or for recreational activities). In recent years, new tools have been tested with the intention of improving cyanobacterial sampling, for example, remote sensing reconnaissance to determine the horizontal distribution of cyanobacteria in freshwater ecosystems (Hunter et al., 2009), or spectrofluorometric probes to reveal the vertical distribution of these cyanobacteria in the water column (Leboulanger et al., 2002). Moreover, these spectrofluorometric probes and other sensors have now been integrated into buoys, to provide real-time monitoring of cyanobacteria in freshwater ecosystems (Le Vu et al., in press).

However, despite the great potential interest of these tools, their cost will remain prohibitive for their routine use in the foreseeable future, and most of the monitoring programs worldwide for the survey of cyanobacteria will continue to be based on more conventional methods for some years to come. Taking discrete samples of various volumes of water taken from the shoreline of ecosystems is probably the method one most often used in studies. Unfortunately, as a result of spatial and temporal differences in the distribution of cyanobacteria, this approach can often provide a very poor estimation of cyanobacterial abundance and, consequently, of the associated health risk. We therefore need to devise simple sampling strategies for the low cost monitoring of cyanobacteria in shallow lakes. In an attempt to do this, we performed intense spatiotemporal monitoring of cyanobacteria in a shallow lake known to experience cyanobacterial blooms every year.

2 Materials & Methods

2.1 Study site

This study was performed in a shallow lake named Place (0.08 km^2, 2.5 m max depth, 45°43'N, 4°14'E) located in the plain of Forez (Central France), (Fig. 1). This lake is used for extensive fish production and its trophic status is eutrophic to hypereutrophic (OCDE, 1982). *Microcystis aeruginosa* blooms occur every summer.

2.2 Data acquisition

2.2.1 Sampling strategy and cell counting

In order to assess the variations in the horizontal distribution of cyanobacteria in this pond, we monitored six sampling points located around the lake at one meter from the shore (V1-V6; Fig. 1). The water depth in each of these sampling points was around 1 meter. Samples were taken every two days, between 09:00 and 10:00 a.m., from early June 2008 to early October 2008. The first 40-centimeters of the water column were sampled using a watersampler (Uwitech, Austria). This water sample was shacked and then divided into two 1-L bottles, one liter being stored at room temperature with Lugol's iodine solution, and the other at 4°C.

In order to evaluate the vertical distribution of cyanobacteria in the water column, we performed a 22-hour survey (from 4:30 p.m. August 4, 2009 to 2:30 p.m. August 5, 2009), of the variations of cyanobacterial biomass at five sampling points (A-E; Fig. 1) using a BBE Algaetorch (Moldaenke, Germany). This torch is based on the same principle as the BBE spectrofluorometric probe (Beutler et al., 2002), but provides only an estimation of the concentrations of cyanobacteria and total chlorophyll in water. Every hour, the torch was immersed to a depth of 20 centimeters at the five sampling points, and triplicate measurements were performed in each point.

The cyanobacterial cell concentrations were estimated using a Nageotte cell and an optical microscope, as described in Brient et al. (2008). For each rectangular area, we counted at least 400 cells of each cyanobacterial species.

2.2.2 Meteorological data

The speed and direction of wind during our study were obtained from the Metéo France meteorological station at St Etienne-Bouthéon (4°18'E – 45°32'N). The wind direction rose for this station is given in Supplemental Figure 1, and shows that the two dominant wind directions were NW and SE. The direction of winds blowing from 240° – 60° was classified as NW, and that of winds blowing from 60° – 240° as SE.

2.3 Data analysis

The spatial distribution of cyanobacteria in the lake was represented using Surfer (v. 7.0, Golden Software Inc.), and statistical analyses (Wilcoxon test, Spearman correlation) were performed using the R package version 2.10 (R development core Team, 2010).

3 Results

3.1 Change over time in the population dynamics of the two dominant cyanobacterial species

Two cyanobacterial species, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*, dominated the phytoplankton community during the summer of 2008. The population dynamics of these two species displayed very contrasting patterns (Fig. 2). The population dynamics of *Microcystis aeruginosa* was characterized by a steady increase in the cell abundance from June to August, apart from a brief dip in the middle of July. The maximum population was reached on August 21 (264,000 cells/mL), and subsequently the cell concentration remained stable until the end of September, and then decreased in October. In contrast, the population dynamics of *Aphanizomenon flos-aquae* were much more chaotic, with the cell abundance reaching two very high and short-lived peaks in July (400,000 cells/mL on July 17, and 560,000 cells/mL on July 23).

3.2 Influence of sampling frequency on the estimation of the population dynamics

Our assessment of the changing population dynamics of the two cyanobacteria were obtained using a very frequent high temporal sampling regime (every two days), which would not be practicable in the context of normal monitoring programs. In order to evaluate the impact of the sampling frequency, we simulated weekly, twice-monthly and monthly sampling frequencies to our data set. The results of these simulations are shown in Fig. 3 and 4. From this figure, we can see that changes in *M. aeruginosa* cell abundance over time would have been fairly accurately estimated at all these sampling frequencies. Moreover, for all sampling frequencies, the quality of the estimation of the *M. aeruginosa* population dynamics was not influenced by choice of the first sampling date (Fig. 3). In contrast, the population dynamics of *A. flos-aquae* would have been badly or even very badly estimated by using weekly, twice-monthly and monthly sampling frequencies (Fig. 4). We would only have detected both *A. flos-aquae* peaks in one of the three trials testing the weekly sampling strategy, and we would never have detected these peaks with twice-monthly and monthly sampling frequencies.

3.3 Evolution of the horizontal distribution of cyanobacteria in the lake during the bloom

As shown in the video (Supplemental Fig. 2), the horizontal distribution of both cyanobacteria displayed marked variations during the course of the study. Moreover, when the spatial distributions of the two species at the same sampling dates were compared, it could be seen that similar or contrasting patterns in the horizontal distribution of *M. aeruginosa* and *A. flos-aquae* cells would have been found, depending on the dates chosen (some examples are provided in Fig. 5).

In order to obtain a better picture of this spatial variability in the cell concentrations of the two species, we estimated the coefficients of variation in the mean abundance for each sampling date and for each species from the results obtained at the six sampling points (Fig. 6). These coefficients were usually higher for *A. flos-aquae* than for *M. aeruginosa* (Wilcoxon test, p=-3.25.10^{-05}), suggesting that the horizontal distribution of *A. flos-aquae* was more variable. Finally, there was no correlation (Spearman coefficient) between the coefficient of variation and the mean cell abundance for *Aphanizomenon*, and only a weak correlation was found for *Microcystis* (Spearman coefficient, p=0.003 r =-0.4; Supplemental Fig. 3).

In order to find out whether wind speed/direction could account for the variations in the horizontal distribution of cyanobacterial cell abundance in the lake, we recorded in a first time, for each species and for each sampling date, the sampling point (out of the six) at which the highest cell abundance was detected. We then constructed a table in which we related these findings to the wind direction and speed in the five hours before the sampling, knowing that only data with wind speed values ≥2.0 m/s were taken into consideration. For *M. aeruginosa*, the detection of the highest cell abundances in the southernmost sampling points V2 and V3 were associated with winds blowing from the NW (Table 1), whereas those at the V1 and V4 sampling points were more surprisingly associated with winds from the SE. High cell abundances in the northern most sampling points V5 and V6 were equally associated with winds from NW and SE. For *A. flos aquae*, the results were more complicated, and no obvious link could be seen between the direction of the wind and the distribution of the cyanobacteria (Table 1). The same analyses were performed by taking into account the wind data one and two days before sampling (instead 5-10 hours before sampling), but no obvious relationship was detected (data not shown).

3.4 Influence of the number of sampling points on the estimated cyanobacterial cell abundances in the lake

The cyanobacterial cell abundances in the shallow lake were estimated by calculating the average value for the six sampling points (see Fig. 1). In order to determine the number of sampling points required to obtain a good estimation of cyanobacterial cell abundances in the lake, we compared the estimations of cell abundance based on using samples from just one, two, three, four or five sampling points with that based on all six. To do this, we calculated the correlation coefficients (Spearman) between the estimations based on the six sampling points and those based on one to five sampling points for each species (Fig. 7). We considered all possible combinations of points, and the results are classified in the figure on the basis of increasing order of r values within each combination of groups. For both species, we found that the estimations of cell abundances based on only one or two sampling points were generally rather badly correlated with those obtained using all six sampling points. On the other hand, it appeared that good correlations (around or > 0.9) were obtained when at least three sampling points were used, but also that the variations due to the choice of the sampling points was still considerable when only three sampling points were used.

In order to find out which combinations of sampling points provided the best results when only two or three sampling points were used, we classified all the possible combinations of points. To do this, we added the rank of each combination of sampling points obtained for the two species (*M. aeruginosa* and *A. flos-aquae*). From Figure 8, we can see that the best estimations obtained using only two or three sampling points were provided by combinations in which the sampling points used were on the shore opposite to the prevailing wind direction over the lake.

3.5 Diel variations in the subsurface cyanobacterial biomass in the lake

Finally, we carried out a 24-hour estimation of the variations in the total cyanobacterial biomass in the subsurface water (20 cm depth) of the lake, at five sampling points using the

BBE torch (A-E, see Fig. 1). As shown in Fig. 9, there was a steady fall in the cyanobacterial biomass at all sampling points during the afternoon and evening, and conversely an increase late at night and in the morning. Moreover, the differences in biomass between the five sampling points were smaller during the night than during the day, as was the standard error (three measurements per sampling point). A multidimensional scaling analysis performed on the same values confirmed these observations, with all the night sampling times being grouped together, whereas the sampling times during the day were much more scattered (Data not shown).

4 Discussion

As far as we are aware, this is the first attempt to investigate the influence of sampling strategies on the evaluation of spatial and temporal variations in cyanobacterial abundances in shallow lakes, which constitute unstable and complex ecosystems. These lakes are used by humans for numerous activities, including recreational activities and the supply of drinking water, which makes the monitoring of cyanobacteria in such ecosystems of particular importance, especially as part of the evaluation of the health risks linked to cyanobacterial blooms and their toxins. Sampling strategy is also very important in the context of basic studies, because the quality of sampling has a major impact on the quality of the final results.

In this study, we found that the sampling frequency required to obtain a good estimation of the temporal evolution of the cyanobacterial abundance depends on the blooming species, *M. aeruginosa* or *A. flos-aquae*. Twice-monthly or monthly sampling provided good results for *M. aeruginosa*, whereas this was not often enough to monitor the chaotic population dynamics of *A. flos-aquae*. These findings are in contradiction with the recommendations of Codd et al. (1999), who proposed weekly or a twice-monthly sampling for species that do not form scum (*A. flos-aquae* for example), and more frequent sampling for scum-forming species (such as *M. aeruginosa*), because they can display more rapid changes in concentration. On the other hand, in agreement with these authors, our findings also demonstrate that a reactive approach to cyanobacterial sampling is called for, and that appropriate monitoring programs must be devised for each ecosystem based on what is known about how these systems function. It is clear that sampling only once or twice a month can lead to a very considerable under-estimation of cyanobacterial concentrations, and thus of the health risks associated with the bloom. As a result, a weekly sampling frequency seems to be required for cyanobacteria in small freshwater ecosystems.

Our data on the variability of the spatial distribution of cyanobacteria in the lake indicate that at least three sampling points were needed to obtain a good estimation of the abundance, based on a comparison with estimations based on six sampling points. It appeared also that if only three sampling points are used, the choice of the location of these sampling points is very important for the quality of the estimation. The most reliable results were obtained using sampling points located on the opposite side of the lake shore to the main axis of the wind direction, and that adding more sampling points reduces the impact of the choice of the location of the sampling points. Such horizontal variability in the distribution of cyanobacteria has been previously documented for many ecosystems, and also for many cyanobacterial species. For example, in a recent study, Briand et al. (2009) showed that the spatial distribution of *M. aeruginosa* in a large freshwater reservoir on a given date could vary from 7.10^3 cells/mL to 2.10^8 cells/mL, depending on the location of the sampling points in the reservoir. Many factors and processes can influence the horizontal distribution of cyanobacteria in a freshwater ecosystem. Among them, wind and surface currents seem to have the greatest impact. For example, the distribution of *Microcystis* spp. in lake Taihu (see the review paper of Qin et al., 2010) and in Lake Ontario (Hotto et al., 2007) is clearly influenced by both winds and currents. Similarly, Moreno-Ostos et al. (2009) have shown that in a Spanish reservoir currents have a marked effect on the distribution of cyanobacteria, and more globally on the phytoplankton community. In this study, we found that the horizontal distribution of *M. aeruginosa* in the lake was influenced more by wind direction than that of *A. flos-aquae*. This could be explained by the fact that *M. aeruginosa* colonies are located at the surface of the lake at the end of the night, and thus are more subjected to the influence of the wind than *A. flos-aquae* filaments, which are distributed over the entire water column. We found also that two sampling points in the lake (V5 and V6) were less influenced by wind direction that the others. This could be explained by the fact that these two sampling points are protected from the influence of winds blowing from the NW by an embankment located in the North part of the lake. Finally, we also demonstrated that in such a small lake, the impact of wind occurred at the scale of a few hours, in contrast to the previous findings of Welker et al. (2003) showing that the distribution of cyanobacteria was influenced by winds that had been blowing one or two days earlier.

In addition to this variability in their horizontal distribution; the vertical distribution of cyanobacteria was also variable. Indeed, during the 24 h for which we used the BBE Torch to monitor the concentrations of cyanobacteria, we found that they were lower in the subsurface layer early at night than during the day. The greatest variations in biomass were recorded during the daytime, both at the scale of one sampling point when the three measurements were compared, and at the scale of the five sampling points monitored during this study. These findings also suggest that several sampling points are necessary to obtain an accurate assessment of the cyanobacterial biomass and that integrated sampling of the first meter of the water column reduces the variability in the estimation of the biomass due to the position of cyanobacteria in the water column. This finding is consistent with data reported by Ahn et al. (2008) showing that an integrated method was the most appropriate sampling method for

Oscillatoria and *Microcystis* blooms. The causes of these variations in the position of cyanobacteria in the water column have been studied for different species. Several papers (Porat et al., 2001; Rabouille and Salençon, 2005; Rabouille et al., 2005; Visser et al., 2005; Walsby, 1994) have shown that migrations of cyanobacteria in the water column are probably due to the dynamics of the carbon-reserve metabolism, and are strongly influenced by light, temperature, and water mixing.

From all these findings, guidelines should be proposed for the monitoring of cyanobacteria in shallow lakes Codd et al. (1999) propose that the choice of sampling frequency and the choice of the number and location of the sampling sites should depend on the purpose of monitoring. For example, sampling near public bathing sites was recommended in freshwater ecosystems used for recreational activities. However, this strategy might generate data relevant only to the immediate vicinity of the bathing area, which do not reflect the global distribution of cyanobacteria in the lake. This is especially true when this distribution is very varied, and could make it difficult to prevent or manage blooms. On the basis of our findings, we proposed a different sampling strategy, which does not depend on the purpose of the monitoring. In order to minimize the cost of the cyanobacteria survey, twice-monthly sampling could be the norm for monitoring, but only if it is complemented by regular visual surveys. Changes in the appearance of the water (e.g. its color) between two successive dates would lead to an immediate increase in the sampling frequency. If it is not possible to carry out this visual survey, only a weekly sampling strategy can ensure that a sporadic cyanobacterial bloom is not missed. With regard to the number of sampling points, we found that at least three sampling points were necessary to obtain an accurate assessment of the cyanobacterial biomass (based on comparison with six sampling points). However, even when three sampling points were used, we found that the choice of the location of the sampling points was also very important (Fig. 8), even though the lake was fairly rectangular in shape and its perimeter small (around 1.3 Km). These findings suggest that for large lakes and also for lakes with a more complex shape, a large number of sampling points would be necessary to obtain a good estimation of the cyanobacterial abundance. Clearly such sampling is time consuming and expensive. One way to reduce these costs would be to collect a large number of samples and then pool equal volumes of these samples in the same flask, before carrying out a single analysis. In this study, as in most of the monitoring programs performed in small lakes, all samples were taken from the shoreline of the lake. This kind of sampling is suitable for small lakes, but it has been shown that for large lakes (Rogalus and Watzin, 2008) shoreline sampling may miss early warning signs of bloom development, and also lead to the overestimation of the concentration of microcystins, when compared to data obtained from offshore samples. For bigger lakes, therefore, the sampling strategy must include offshore samples.

Different programs worldwide are testing alternatives to water sampling for the monitoring of cyanobacteria in freshwater ecosystems. Two main approaches have been investigated. The first one is based on the use of remote sensing, which has long been in use in marine ecosystems (see for example Bracher et al., 2009). In freshwater ecosystems, the paper of Hunter et al. (2008) has shown the potential of high resolution images for the assessment of the spatial distribution of *M. aeruginosa* in a shallow eutrophic lake. However, the cost of these images and the impact of meteorological conditions are limiting factors for envisaging the use of this tool in routine cyanobacteria monitoring programs. One alternative, lower-cost solution could be based, in the future, on the use of drones to take aerial photographs of freshwater ecosystems, but these tools are still in development. Moreover, they will be only useful for cyanobacterial species that live in the surface water of lakes.

The second way of monitoring of cyanobacteria without sampling the water being investigated is the use of buoys equipped with a variety of sensors, including, for example, a submersible spectrofluorometer to quantify the biomass of the cyanobacteria. This kind of tool permits the real-time monitoring of phytoplankton, including cyanobacteria, as shown for example in the paper of Le Vu et al. (in press). The two obstacles to their use in routine cyanobacteria monitoring programs are the high price of these systems, and the fact that they only provide estimations for one sampling point. Despite this, the possible use of such buoys, combined with the spatial monitoring of cyanobacteria by water sampling looks very promising for surveying cyanobacteria in freshwater ecosystems.

5 Conclusion

The sampling of cyanobacteria in freshwater ecosystems is a hot topic, in particular in the context of programs for surveying these toxic microorganisms in ecosystems used for the production of drinking water or for recreational activities. Paradoxically, fewer studies deal with the impact of sampling strategies on the estimation of cyanobacterial cell abundances in freshwater ecosystems. In this study, we demonstrate that the choice of sampling strategy can lead to very different estimations of the cell abundances of two blooming species in a shallow lake and also that, depending on the cyanobacterial species involved, different sampling strategies are required to obtain a good estimation of their population dynamics. All these findings suggested that monthly or twice-monthly sampling strategies at just one sampling point do not allow to provide an accurate estimation of cyanobacterial abundances, and thus of the health risks associated with the presence of toxic species in aquatic ecosystems. Moreover, although promising new technologies are being developed for monitoring freshwater cyanobacteria, their cost and some other drawbacks mean that at present they cannot replace water sampling, which will remain the basis of most of these monitoring programs for the foreseeable future.

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Table 1: Relationship between wind direction and high cell abundance recorded for *Microcystis aeruginosa* and *Aphanizomenon flos-aquae* at the different sampling points. We

Fig. 1: Geographical location of the study site in France (left), and of the sampling points in the lake (right)

Fig. 2: Changes over time of the concentrations of *Microcystis aeruginosa* (top) and *Aphanizomenon flos-aquae* (bottom). These concentrations were estimated by calculating the average cell count for the six samples at each date. The error bars indicate the standard deviation.

Fig. 3: Simulation of the change over time of *Microcystis aeruginosa* cell concentrations found using a weekly (top), twice-monthly (middle) or monthly sampling frequency (bottom), with lags for the first sampling day of zero days (–), 2 days (--) and 4 days (….) comparing to our first sampling day. The gray curve corresponds to the reference data.

Fig. 4: Simulation of the change over time of the biomass of *Aphanizomenon flos-aquae* found using a weekly (top), twice-monthly (middle), or monthly sampling frequency (bottom), and with lags for the first sampling day of zero days (–), 2 days (--) and 4 days (….) comparing to our first sampling day. The gray curve corresponds to the reference data.

Fig. 5: Spatial distribution of two cyanobacteria, *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*, in the lake at four sampling dates (July, 9, 17 & 23; August, 8)

Fig. 6: Change over time in the coefficients of variation of the mean cell abundances of *Microcystis aeruginosa* (black triangle) and *Aphanizomenon flos-aquae* (white square) estimated at all six sampling points.

Fig. 7: Spearman correlation values between *Microcystis aeruginosa* (top) and *Aphanizomenon flos aquae* (bottom) cell abundances estimated from the mean values for all six sampling point values, and those estimated from only one, two, three, four or five of these six sampling points.

Fig. 8: Location of the sampling points providing the best (left) and worst (right) estimations of cyanobacterial cell abundances, compared to estimations based on six sampling points. We give the combinations for two (top) and three (bottom) sampling points. The polar plot shows the direction of the maximum daily wind speed during the study. The different line types permit to distinguish the two best or the two worst combinations of sampling points, using two or three sampling points.

Fig. 9: Cyanobacterial biomass in the subsurface water of the lake over a 24-hour period at five sampling points (♦ point A, ■ point B, ▲ point C, × point D, and ◊ point E). The error bars indicate the standard deviation.

Supplemental Figure 1. Distribution of the wind directions at the St-Etienne-Bouthéon meteorological station during this study (June, 13 to October, 10, 2008). The curve and the bars indicate respectively the mean speed and the occurrence per hour of the wind in each direction.

Supplemental Fig. 2. Evolution of the spatio-temporal distribution of *Microcystis aeruginosa* (left) and *Aphanizomenon flos aquae* (right) in the lake during our study (the scale is the same than in Fig. 5).

Supplemental Fig. 3. Relationship between the cell concentration and the coefficient of variation for *Microcystis aeruginosa* (top) and *Aphanizomenon flos-aquae* (bottom)