The advantages of flow cytometry in comparison to fluorometric measurement in algal toxicity test

Prednosti merjenja s pretočno citometrijo v primerjavi s fluorimetričnim merjenjem v strupenostnih testih z algami

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Abstract: Fluorometric measurements in algal toxicity tests are very commonly used as surrogate parameters for algal biomass. Although, fluorometry is a powerful technique, we have demonstrated that it is not suitable for determination of toxic effects of chemicals, which alter the fluorescence spectra. We recommend the flow cytometry as the best technique for detecting algal and/or cyanobacterial cell count and fluorescence per cell. Flow cytometry has many advantages: little volume of algal/cyanobacterial sample required, suitable also for little algal/cyanobacterial cells, distinction between live and dead cells. Furthermore, flow cytometry reveals the early changes in fluorescence spectra as a consequence of the specific chemicals presence or stress, even though the cell count is not yet affected (an early marker for ecotoxicology testing).

Keywords: algae, cyanobacteria, ecotoxicity test, fluorescence, flow cytometry

Izvleček: V strupenostih testih z algami se zelo pogosto izvajajo fluorometrične meritve kot nadomestni parameter za biomaso alg. Čeprav je fluorimetrija zelo uporabna tehnika, smo pokazali, da ni primerna za določanje strupenih učinkov kemikalij, ki spremenijo spekter fluorescence. Za določanje števila alg in/ali cianobakterij in njihove fluorescence na celico priporočamo uporabo pretočne citometrije. Uporaba pretočne citometrije za zaznavanje alg in/ali cianobakterij ima mnogo prednosti: majhni volumni vzorca alg/cianobakterij, primerno tudi za majhne celice alg/cianobakterij, razlikovanje med živimi in mrtvimi celicami. Poleg tega pretočna citometrija razkrije zgodnje spremembe v spektru fluorescence, ki so posledica prisotnosti specifičnih kemikalij oz. stresa, čeprav število celic še ni spremenjeno (zgodnji marker v ekotoksikoloških testiranjih).

Ključne besede: alge, cianobakterije, ekotoksikološki test, fluorescenca, pretočna citometrija

Introduction

Many toxicity tests and standards are used to describe the water quality. Frequently algal toxicity tests are used because of their relative toxicity simplicity and low price (ISO 8692, 2012; OECD TG 201, 2011). The purpose of these tests is to determine the effects of a substance on the growth of freshwater microalgae and/or cyanobacteria. It is not used strictly for toxicity testing of pure substances but also for mixtures of chemicals, rapid screening of waste water, etc. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure time period. From the average specific growth rate recorded in a series of test solutions, the concentration bringing a specified inhibition of growth rate (e.g. 50%) is determined and expressed as the ECx (e.g. EC50). In ecotoxicity tests the EC10 is used very often to replace LOEC/NOEC values (Shieh at al. 2001, Warne and Rick 2008).

The principle of this test is based on exponentially growing algae and/or cyanobacteria exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed. As a result the reduction of growth in a series of algal cultures exposed to various concentrations of a test substance is observed. The response is evaluated as a function of the exposure concentration in comparison with the unexposed control cultures. For full expression of the system response to toxic effects, unlimited exponential growth under nutrient sufficient conditions and continuous light for a sufficient period of time is provided to the cultures (Oecd tg 201, 2011).

Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. The algal biomass in each flask is determined at least daily during the test period. However, dry weight is difficult to measure and therefore surrogate parameters are used: cell counts (most often), cell volume, fluorescence, optical density, etc. A conversion factor between the measured surrogate parameter and biomass should be known (Oecd tg 201, 2011). Measurement of biomass is done by manual cell counting by microscope or an electronic particle counter (by cell counts and/or biovolume). Alternative techniques e.g. flow cytometry, *in vitro* or *in vivo* chlorophyll fluorescence (Mayer at al. 1997; Slovacey and Hanna 1997), or optical density can be used if a suitable correlation with biomass can be demonstrated over the range of biomass occurring in the test.

There are many occasions where microscope counting for biomass measurements is very complicated because of the shape, granularity or other specific features of the cells; e.g. small cells of cyanobacteria *Synechococcus leopoliensis* are distributed on many different vertical levels in the counting chamber. Therefore it is impossible to count them accurately under the light microscope. Standards recommend electronic particle counters equipped for counting particles down to a size of approximately 1 µm, but also *in vitro* fluorometric measurements are applicable.

Fluorimetric measurements in algal toxicity test can be very useful, since algae and cyanobacteria possess photosynthetic pigments. The fluorometer emits an excitation light at a particular wavelength (approximately 430 to 470 nm) that causes the chlorophyll (CHL) *a* to fluoresce at another wavelength (approximately 650 to 700 nm). The concentration of CHL *a* is proportional to the amount of CHL *a* fluorescence emitted. All algae contain CHL *a*, but there are many kinds of algae and especially cyanobacteria with distinctly different accessory pigments that fluoresce at different wavelengths. Some accessory pigments such as CHL *b* and CHL *c* fluoresce within the same wavelength and may influence the CHL *a* determination (Hambrook-Berkman and Canova 2007).

In this article we demonstrate the method for accessing the biomass with flow cytometry, which gives us even more information than "classic" fluorescence measurements or electronic particle counter. In our study only 70 µl of algal/cyanobacterial sample is used for flow cytometry measurement and we get information about two parametrs simultaneously: cell count and fluorescence spectra. Furthermore, we demonstrate that some chemicals alter the fluorescence spectra of algae. In these cases only flow citometry measurements give us accurate estimation of biomass in contrast to fluorometric measurements, which are not applicable at all.
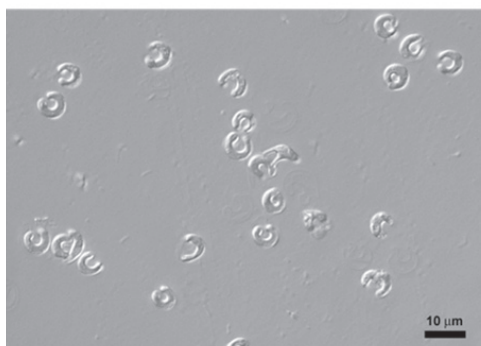
Materials and methods

Cyanobacterial and algal species

Algal growth inhibition test were conducted according to the OECD TG 201 (2011). Two phytoplankton species were selected for all the experiments: green algae *Pseudokirchneriella subcapitata* SAG 61.81 algae collection and cyanobacteria *Synechococcus leopoliensis* SAG 1402-1 (Fig. 1), from the SAG (Sammlung von Algenkulturen Univerziätt Göttingen).

interval $5*10^3$-$5*10^4$ cells/ml for *Pseudokirchneriella subcapitata* and $5*10^4$-$5*10^5$ cells/ml for *Synechococcus leopoliensis*, in order to achieve stabile exponential growth. The composition of the growth medium is defined in OECD TG 201, Annex 3 (2011). The duration of all tests was 72 h. The flasks were incubated at 24°C under cooled lamps Sylvania GRO-Lux F 18 W/GRO-T8 with continuous light (24h) and shaking (GFL 3017, Germany). Light intensity at 80-120 $\mu Em^{-2}s^{-1}$ was determined with Delta-T Logger (Delta-T Devices Ltd., UK) equipped with QS Quantum

Pseudokirchneriella subcapitata

SAG 61.81 green algae

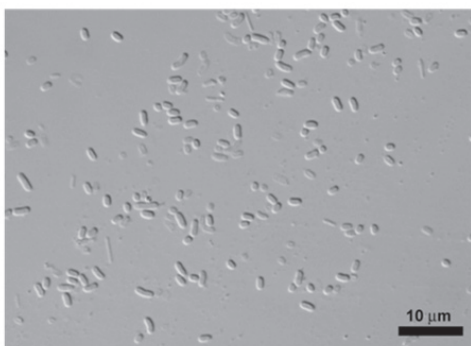**Synechococcus leopoliensis**

SAG 1402-1 cyanobacteria

Figure 1: Green algae (left) and cyanobacteria (right) used for the algal toxicity tests in this study.
Slika 1:   Zelene alge (levo) in cianobakterije (desno), ki smo jih uporabljali za strupenostne teste z algami v tej študiji.

Test design

Algal or cyanobacterial strains were cultivated for several generations in a defined medium containing a range of 5–6 concentrations of the test sample and inoculums of exponentially growing cells. The test batches were incubated for a period of 72 h during which the cell density in each test solution was measured at least every 24 h. Inhibition was measured as a reduction in specific growth rate relative to control cultures grown under identical conditions.

Test was performed in glass Erlenmeyer flasks at least three times in triplicates. The culture volume at the beginning was set to 20 ml. Biomass density at the beginning of the test was within the

sensor. Small fractions were taken away daily for microscopic examination, fluorescence measurement or flow cytometry (not more than 1 ml).

Test substances

Three cytostatics were tested: cisplatin (CP) from Sigma-Aldrich (Seelze, Germany), etoposide (ET) and imatinib mesylate (IM) from Santa Cruz Biotechnology, Inc., US. All cytostatics and their containers were disposed as hazardous waste. Concentrated stock solutions were prepared, stored in refrigerator and used within 2 months. Stock solution for IM was aliquoted and stored in a freezer for maximum 2 months.

Biomass determination

Microscopic examination of algae and/or cyanobacteria samples, closely observing the cell morphology changes, was done at the beginning and at the end of each experiment. For the determination of biomass samples of algae and/or cyanobacteria were analysed with: (a) cell counting under inverted light microscope, (b) flow cytometry and (c) *in vitro* fluorescence determination:

(a) Cells were closely examined for visual cell abnormalities and counted under a light inverted microscope (Nikon Eclipse TE300) using Bürker Türk haemocytometer. The microscope was equipped with a digital camera. Cells were measured with software Lucia (System for Image Processing and Analysis LUCIA 4.6, Laboratory Imaging Ltd.).

(b) Flow cytometer (BD FACSCalibur, USA) was used for the assessment of cell count. Laser sensors FL3 (with Band Pass Filter at 620nm) and FL4 (with Band Pass Filter at 675nm) were used for *P. subcapitata* and FL4 for *S. leopolinesis*. The volume of sample was 70 µl. Flow rate was set to 65 µl/min. The evidence of correlation between cell concentration using different methods was evident from the calibration curve – "flow cytometer cell count" vs. "microscopically determined cell count" for more than 100 simultaneous measurements/counts (data not shown). Furthermore, we have simultaneously count the external control (red and blue microsphaeres from EQAT – External quality assessment trials Phytoplankton, red = external control for cyanobacteria *S. leopoliensis*, blue = external control for green algae *P. subcapitata*).

(c) Changes in chlorophyll fluorescence were followed in the red (l_{em} 680 nm) using l_{ex} 440 nm, where the chlorophyll *a* has a significant absorption peak (Lee at al. 1994).

Before measuring fluorescence, samples were dark-adapted for approx. two hours. The fluorescence was measured using Synergy Mx Monochromator-Based Multi-Mode Microplate Reader; excitation and emission slit widths were 20 nm, shaking before measurement was: 5 sec at 24˚C. The measurement were normalised to the control at the beginning of the experiment, since the cell count in controls varies between different experiments.

Statistical evaluations

Data were analysed (software Prism 5, Graph-Pad Inc.) as measurement from each individual flask (pooled together) rather than means of replicates, in order to extract as much information from the data as possible (suggested in OECD TG 201, 2011). For dose-response graphs the nonlinear regression model was used ("log(inhibitor) vs. response – variable slope", no constrains). For graphs with % of inhibition vs. concentration of cytostatic (and EC10, EC20 and EC50) assessment, the nonlinear regression model was used ("log (inhibitor) vs. response – variable slope", with constrains for shared value for bottom and top).

Results and discussion

The fluorometric measurements are based on properly selected excitation and emission wavelength to detect the fluorochromes of algae and/or cyanobacteria. Green alga *P. subcapitata* and cyanobacterium *S. leopoliensis* demonstrated weak correlation between fluorometric measurements and microscopically determined cell count for more than 160 simultaneous measurements/counts (calibration curves on Fig. 2). The average correlation factor was quite low 0.68. Since the cell density may influence the calibration curve, we have divided results into "low and high density" cultures (compare A and B on Fig. 2). There is no significant influence of cell density on fluorescence in the frame of exponential growth phase.

On the other hand, flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5 µm to 40 µm diameter. Flow cytometry in our study showed very consistent results with the "standard" red and blue microsphaeres, when compared to microscope counting (Fig. 3).

One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This is in contrast with spectrophotometry in which the percent of specific wavelengths of light is
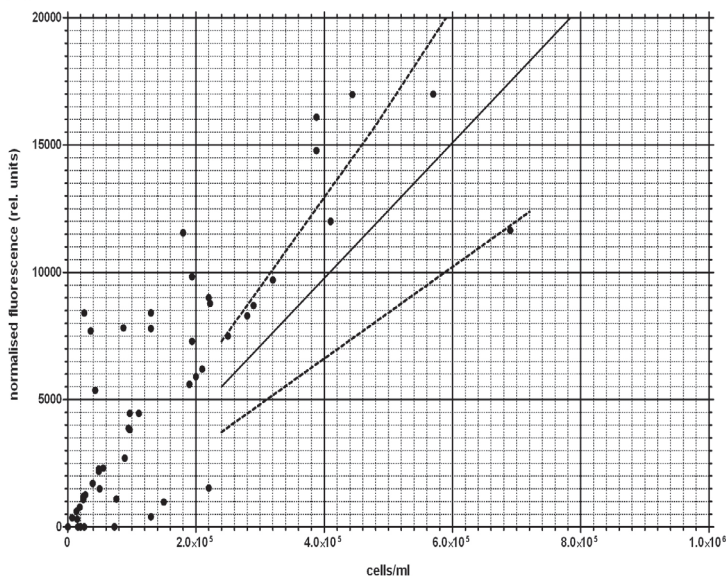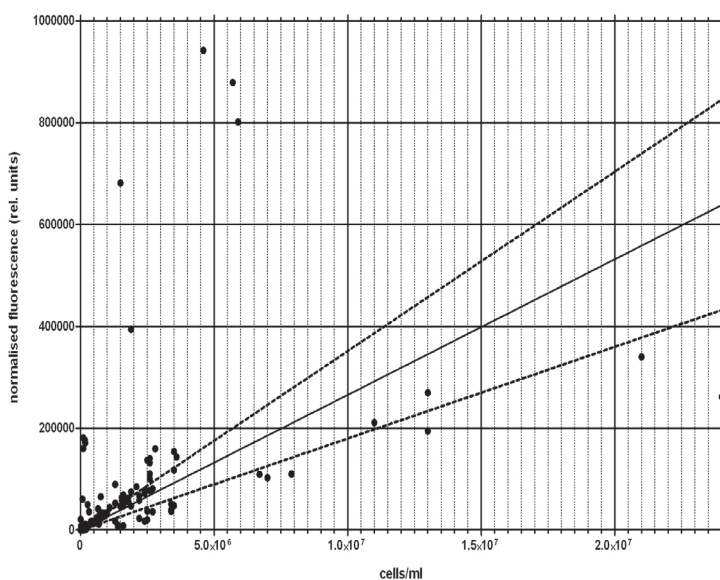
(A) low density cultures

(B) high density cultures

Figure 2: The cell concentration on the calibration curve - the fluorimetric measurements (axis y) and microscopically determined cell count (axis x) show no evident correlation in low (A) or high (B) density cultures of P. subcapitata.

Slika 2: Koncentracija celic na umeritveni krivulji – fluorometrične meritve (os y) in mikroskopsko določeno število celic (os x) ne kaže očitne korelacije niti pri nizki (A) niti pri visoki (B) gostoti kultur P. subcapitata.
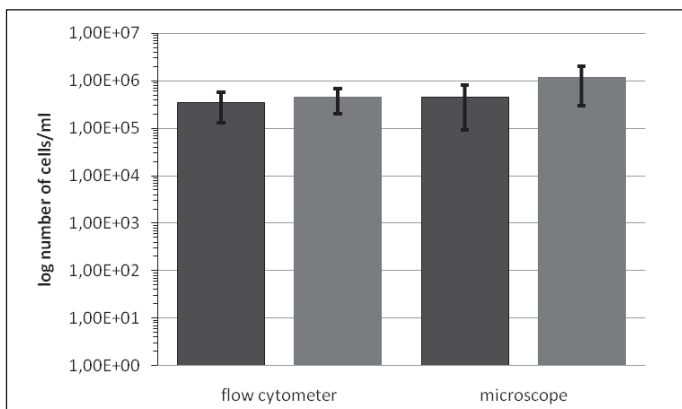
Figure 3: The comparison of counting method with flow cytometer vs. microscope counting of the external control (microsphaeres, EQUAT, black colour = control for cyanobacteria *S. leopoliensis*, gray colour = control for green algae *P. subcapitata*).

Slika 3: Primerjava števne metode s pretočnim citometrom vs. mikroskopsko štetje zunanje kontrole (kroglice EQUAT, črna barva = kontrola za cianobakterijo *S. leopoliensis*, siva barva = kontrola za zeleno algo *P. subcapitata*).
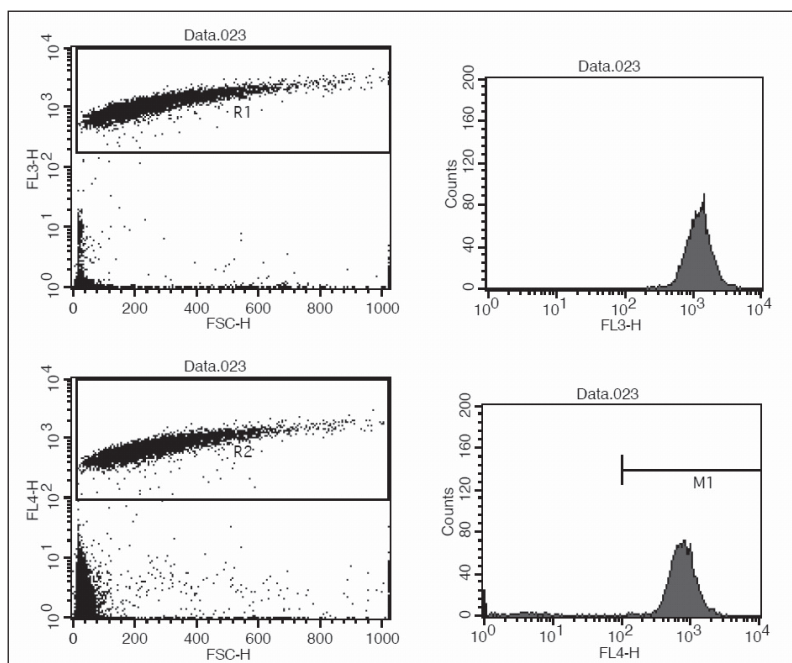

Figure 4: Flow cytometry dot-blot histogram (left) of green algae P. subcapitata and fluorescence spectra (right) of gated events (for gate R1 we used laser sensor FL3 = top and for gate R2 laser sensor FL4 = bottom, counting interval = M1).

Slika 4: Točkovni histogram pretočnega citometra (levo) zelene alge P. subcapitata in spekter fluorescence (desno) uokvirjenih dogodkov (za okvir R1 smo uporabili laserski senzor FL3 = zgoraj in za okvir R2 laserski senzor FL4 = spodaj, interval štetja = M1).
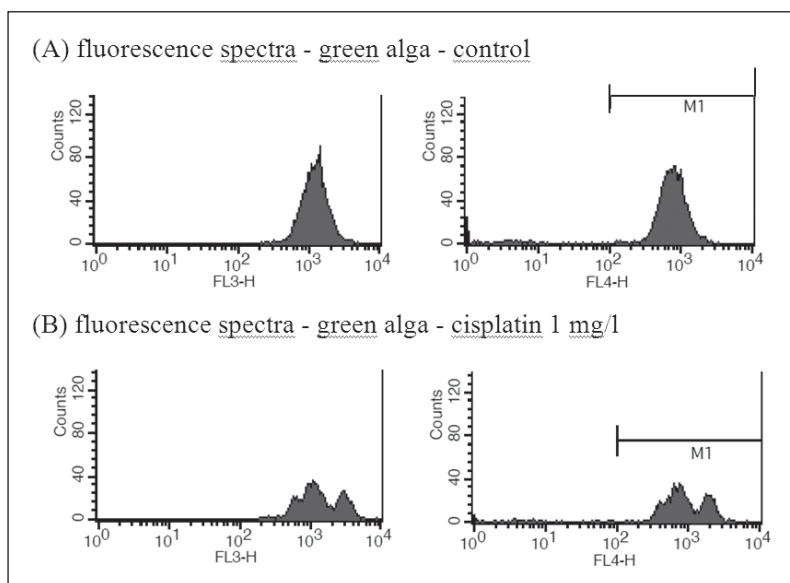
Figure 5: Flow cytometry histogram (with laser sensor FL3 = left and FL4 = right, counting interval = M1) show-
ing shift in fluorescence spectra of green algae P. subcapitata 24 hours after the addition of cisplatin at
1 mg/l (B) in comparison to control (A), even though the cell count is not yet affected.

Slika 5:   Histogram pretočnega citometra (z laserskim senzorjem FL3 = levo in FL4 = desno, interval štetja =
M1) kaže spremembo v spektru fluorescence zelene alge P. subcapitata 24 ur po dodatku cisplatina v
koncentraciji 1 mg/l (B) v primerjavi s kontrolo (A), kljub temu, da učinek na koncentracijo celic še ni
bil opazen.

measured for a bulk volume of sample. To study toxic changes in *P. subcapitata* and *S. leopoliensis* with flow cytometry we have detected the size and granularity (two parameter histogram or Dot Plot, Fig. 4 – left) and fluorescence spectra of gated events (Fig. 4 – right, Fig. 5). Flow cytometer revealed that tested cytostatic cisplatin (CP) altered the fluorescence spectra in green alga, even though the cell count was not affected. Under the same conditions no changes in fluorescence spectra were found in the case of imatinib (IM) or etoposide (ET), data not shown. The fluorescence spectra of selected algae and cyanobacteria may change as a consequence of chemical action, as proved with the flow cytometry in our study with cytostatic cisplatin (Fig. 5) and other chemicals (e.g. Regel at al. 2002). Therefore, we considered fluorometric technique not precise enough and for this reason all the subsequent testing was done with the use of flow cytometry, which is becoming more and more popular (Hashemi at al. 2011). Also fluoro-

metric measurements are quite popular in algal toxicity test (Mayer at al. 1997, Berden-Zrimec at al. 2007, Nguyen-Ngoc at al. 2009), but some chemicals may shift or move the fluorescence peak and one should be very cautious with the interpretation of fluorometric measurement at specific wavelength(s).

There are many advantages of the use of flow cytometer for algal/cyanobacterial detection. As little as 70 µl of algal/cyanobacterial suspension (+70 µl of filtrated distilled water) is enough to accurately determine the cell count and fluorescence spectra in our exponentially growing cultures. Flow cytometry is suitable also for little algal/cyanobacterial cells, which can not be precisely counted under the microscope. It counts only cells which possess active chlorophyll pigments, so it can be used to distinguish between live and dead cells. Flow cytometry reveals the changes in fluorescence spectra as a consequence of specific chemical presence or stress, even though the cell

count is not yet affected. This is the reason why this method can be used for early detection of chemicals which alter the fluorescence spectra as an early marker for ecotoxicology testing.

Povzetek

Fluorometrične meritve v strupenostnih testih z algami se zelo veliko uporabljajo kot nadomestni parameter za biomaso alg. Kljub temu, da je fluorimetrija zelo uporabna tehnika, bi radi izpostavili pomankljivost pri uporabi za določanje strupenih učinkov kemikalij, ki spremenijo spekter fluorescence. Vrh fluorescence se namreč spremeni oz. premakne v druge valovne dolžine, zato detekcija pri določenih valovnih dolžinah (s »klasično fluorometrično metodo«) ni dovolj natančna in posledično ne moremo izračunati pretvorbenega faktorja med merjenim nadomestnim parametrom in biomaso. Pretočno citometrijo priporočamo za detekcijo biomase alg, saj ima mnogo prednosti kot npr. sočasno merjenje števila celic in fluorescence na celico.

Summary

Fluorometric measurement in algal toxicity tests are very commonly used as surrogate parameters for algal biomass. Although, fluorometry is a powerful technique, we would like to point out a disadvantage, when used for determination of toxic effects of chemicals, which alter the fluorescence spectra. Since the fluorescent peak is shifted or moved to other wavelength, detection at specific wavelength (with "classic fluorimetric methods") is not precise enough and the conversion factor between the measured surrogate parameter and biomass can not be determined. We recommend the flow cytometry for detecting algal biomass, since it has many advantages, including synchronous measurement of cell count and fluorescence per cell.

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