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Use of fluorescence information for automated phytoplankton investigation by image analysis

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Automated identification and quantification of algae in microscopic images is a tool that allows high taxonomic resolution with reasonable technical efforts. However, in samples containing various non-algal objects, this is still not a satisfactorily solved problem. We show that autofluorescence information improves discrimination of algae from non-algal objects as well as phycoerythrin (PE) containing algae from others. We analyse the stability of the autofluorescence to estimate its constraints. Cold and dark storage of glutaraldehyde fixed samples maintains autofluorescence sufficiently for 3 weeks. Under repeated excitations, chlorophyll a (Chl a) or PE autofluorescence show an exponential decrease followed by an intermediate maximum. A peak also occurs in emission wavelength ranges without chlorophyll and PE fluorescence. The unspecific autofluorescence causing the peaks is at least partly identical with the blue–green fluorescence (BGF) in plant cells. BGF interferes with identification of algae, thus correction of pigment autofluorescence with such unspecific fluorescence allows a more reliable algal discrimination procedure. A classification scheme for discrimination of Chl a and PE-containing algae shows a high performance in a test with natural samples. Integration of fluorescence and bright-field image information provides a powerful tool for phytoplankton analysis in complex samples.

INTRODUCTION

Detailed investigations of plankton community composition are essential in various fields, e.g. ecology, ecotoxicology and waste water management (Sommer, 1989; da Motta *et al.*, 2002; Hense *et al.*, 2007). Usually, taxonomic investigations on plankton composition are work and time-intensive, thus costly, making integration of automated steps desirable. For algal research, different techniques using composition of photosynthetic pigments such as flow cytometry and (spectro)fluorometry speed the working process considerably, but their taxonomic resolution is low (Schäfer *et al.*, 1995; Franqueira *et al.*, 2000; Millie *et al.*, 2002; Gaevsky *et al.*, 2005; Gregor and Marsalek, 2005; Larson and Passy, 2005). Variation of pigment composition and concentration within different species of a taxonomic group and in dependency of environmental conditions interfere with the results, as well as fluorescing non-algal particles (detritus) (Sieracki *et al.*, 1985, Fischer and Bunke, 2001, Millie *et al.*, 2002, Gregor *et al.*, 2005, Aberle *et al.*, 2006). Thus, instead of taxonomic groups, rather spectral signalling groups are identified, limited to a number of three to five (Aberle *et al.*, 2006). Acquiring microscopic bright-field pictures of algae and subsequent analysis of species by image analysis has a high potential for community structure analysis, as it principally

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enables one to identify and to quantify each algal cell in a sample saving operators working time. Most appropriate image analysis procedures use morphological features (Tang *et al.*, 1998; Walker and Kumagai, 2000; Bayer *et al.*, 2001; Fischer and Bunke, 2001; Gray *et al.*, 2002; Embleton *et al.*, 2003; Culverhouse *et al.*, 2006). However, they have to deal with a number of problems, e.g. high number of different taxa and high number of non-target (non-algae) objects in natural samples. Freshwater samples often contain a high density of detritus particles. Bright-field or phase-contrast information, often used on a base of grey images, rarely give sufficient optical information for satisfactory identification. In cases of high "non-algal density/algal density" ratios, the number of false-positive results often impedes correct algal counting. Therefore, as much as possible information should be gathered by a combination of different methods (Culverhouse *et al.*, 2003). The addition of pigment-dependent features, e.g. in fluorescence images, to the bright-field or phase-contrast images provides such a combination.

The chlorophyll *a* (Chl *a*) autofluorescence in algae enables algae and non-algae particles to be distinguished (Franqueira *et al.*, 2000). Principally, the composition of accessory pigments with different autofluorescence spectra in the algal classes provides a tool for taxonomic separation (Schäfer *et al.*, 1995; Babichenko *et al.*, 1999). However, spectra of algal classes were partly difficult to separate. Therefore, full use of this tool demands a sophisticated technique such as laser and spectral fluorescence analysis, making it unsuitable for routine, cost-effective microscopic image acquisition and analysis.

Nevertheless, a simplified technique for epifluorescence microscopes, with different fluorescence filter combinations for the pigments Chl *a* and phycoerythrin (PE), has the potential to render taxonomically relevant information (Sieracki *et al.*, 1985; Belykh *et al.*, 2006; Ernst *et al.*, 2006).

Although fluorescence has proven its discriminatory potential, some obstacles exist. Relevant fluorescence as well as colour information is carried in algae mainly by photosynthetic pigments (Larkum *et al.*, 2003). In fixed algae, the pigments fade (bleach), depending on environmental factors such as light and temperature. (The terms "fade" and "bleach" have been inconsistently defined in the literature. In this paper, "fade of fluorescence" means a decrease of fluorescence intensity, regardless of the physico-chemical mechanisms.) Repeated excitations, caused, for example, by overlapping excitation areas of different images, as well as scattered light during image acquisition promotes fading. The combined image acquisitions with bright-field and one or more fluorescence excitations, possibly each with more than one magnification (to take the wide size range of algae into account), also leads to repeated light exposure. Furthermore, fading occurs during normal sample storage. Non-algal objects often show fluorescence within the emission spectra of pigments. The intensity per area of this fluorescence increases with the object volume, complicating a discrimination of weak fluorescing (small) algae from large non-algal objects. The mentioned fading of algal pigments enhances this difficulty.

For a reliable use of fluorescence, quantitative understanding about this is needed; however, this is yet hardly available. We thus investigated the following aspects.

- How does the time course of fluorescence fading during repeats of image acquisitions using bright-field and two fluorescence filter combinations (for Chl *a* and PE) influence the image analysis?
- How do temperature and light conditions during storing of the samples change fluorescence and colour properties?
- How can a classification procedure based on fluorometric features for Chl *a* and PE-containing algae been built?
- Is a sufficient separation of Chl *a* and PE-containing objects by such a procedure possible for a range of different samples?

The study was based on a new automatic system for phytoplankton analysis in water samples (Rodenacker *et al.*, 2006). This system, called PLASA (Plankton Structure Analysis), comprises microscopic image acquisition, archiving and evaluation by image analysis. It allows the consideration of bright-field and fluorescence information and contains a number of different morphometric, densiometric, colourimetric and fluorometric features to identify algae taxonomically as exactly as possible. However, as this paper focuses on the potential use of fluorescence information, mainly fluorometric features were considered. In the following section, the PLASA features used are briefly described. For more details, see Rodenacker *et al.* (2006).

METHOD

Microscope

Image acquisition was carried out with an automatic inverse microscope DM IRBE (Leica, Bensheim, Germany) and a 3-CCD colour TV camera KY-F58 (JVC, Tokyo, Japan). Note that the digital camera discriminated the emitted (fluorescence) light in red, green

and blue spectral ranges. The 40× N-Plan objective, n.a. 0.55 was used. The light source for bright-field was a 100 W Halogen lamp and for fluorescence excitation an HBO 103 W/2 mercury short arc lamp (both Osram, Munich, Germany). The automatic focus, the lamp voltage and the stage are controlled by a self-developed procedure, running under the Leica firmware QWIN. The digitized images were stored on the computer disk each with a size of 760×576 pixels. The pixel sizes correspond to 0.316 μm.

Fluorescence filter

In principle, several algal pigments can be identified according to their autofluorescence properties (Schäfer *et al.*, 1995; Richter, 1988; Wetzel, 2001; Walker *et al.*, 2002; Larkum *et al.*, 2003). However, previous experiments showed that the properties of normal epifluorescence microscopy allow reliable discrimination of three object groups:

- Algal groups containing Chl *a and* PE: in freshwater mainly Cyanophyceae and Cryptophyceae. Further groups were Rhodophyceae and Glaucocystophyceae.
- Algae groups containing Chl *a and not* PE: in freshwater mainly Chrysophyceae, Bacillariophyceae, and Dinophyceae. Further groups were Xantophyceae, Phaeophyceae, Eustigmatophyceae and Synurophyceae.
- Objects without chlorophyll (*not* Chl *a*, non-algae objects including dead algae).

According to Schäfer *et al.* (Schäfer *et al.*, 1995) and Larkum *et al.* (Larkum *et al.*, 2003), Chl *a* autofluorescence has an excitation maximum around 430−490 nm and emits light at wave lengths above 665 nm. PE shows an excitation maximum at higher wave length (above 495 nm) and emits primarily around 580−650 nm. Note that a part of the excitation light energy absorbed by PE as well as by other pigments (carotinoids) is transferred to Chl *a* and thus enhances in the Chl *a* autofluorescence. Besides PE, other biliproteins such as phycocyanin, found in different amounts in Cyano-, Crypto- and Rhodophyceae, also emit red light (around 650 nm), but are usually excited at longer wavelengths than PE (Larkum *et al.*, 2003). Previous experiments indicated that these pigments were not suitable for taxonomic discrimination; however, they interfere to some degree with a clear attribution of fluorescence to Chl *a* and PE. For practical reasons, we nevertheless refer to the emitted light as Chl *a* and PE fluorescence.

Two fluorescence filter combinations were used (both Leica):

a. I3: excitation filter BP 450-490, dichroic reflector 510, emission filter LP515;
b. N3: excitation filter BP546/12, dichroic reflector 565, emission filter BP600/40.

From the RGB images of these filter combinations three spectral channels were used (Table I).

Phytoplankton preparation

All phytoplankton samples, taken from a small pond near Munich (Bavaria, Germany), were fixed with about 0.1% glutaraldehyde, sedimented for 24 h at room temperature (about 20°C) in a plankton chamber using the Utermoehl method (Utermoehl, 1958) and analysed using an inverted microscope. The glutaraldehyde concentration was determined in previous tests making the best compromise between structural preservation and minimization of fluorescence intensity loss. After fixation, the samples were stored in a dark coolbox (4°C). The microscope was located in a dark room, so that the light of the halogen and the fluorescence lamp was almost the only illumination of the samples.

Image acquisition with PLASA

Two subsets of images were taken, bright-field and fluorescence, both from the same locations, which is necessary to identify the same objects in both subsets. An autofocus procedure of QWIN was applied at each location using the bright-field mode. Lamp voltage and optical parameters in QWIN (gain, offset, colour correction) for bright-field were set as recommended by Leica, Bensheim, to achieve as bright as possible, but

Table I: Abbreviations of image analysis features used

Abbrev.	Meaning
FLU1	Fluorescence intensity (a.u.); emission in the RED channel of filter set I3, containing Chl *a* fluorescence
FLU2	Fluorescence intensity (a.u.); emission in the GREEN channel of filter set I3, unspecific fluorescence of various substances
FLU3	Fluorescence intensity (a.u.); emission in the RED channel of filter set N3, containing PE fluorescence
FLU1*−FLU3*	Fluorescence intensity of the areas segmented with a RATS (see text) (a.u.); emission in spectral channels as in FLU1-3
CHROM	Chromaticity; (a.u. [0 . . . 100])
HUE	Hue [−180°. . .180°]
IOD	Integrated optical density (a.u.)

not saturated, image with an uncoloured background. The QWIN settings of the fluorescence images were chosen as for bright-field, but without colour correction. Twelve fluorescence pictures were integrated to get visible and smooth fluorescence. Whereas the exposure time per image in halogen light varies mainly depending on the velocity of the automatic focus (mean about 45–60 s), the fluorescence exposure was about 2–3 s per filter.

Image analysis with PLASA

Image analysis was carried out with PLASA, which was written using the IDL software (ITTvis, Boulder, USA), including colour and shading correction by white images. Images collected were automatically segmented and processed. Segmentation was performed on the bright-field images and applied to all channels of the fluorescence images. To reduce influences of progressing fading, all segmentation results were rigorously visually controlled and corrected if necessary.

The following features were analysed (an overview of the features used and their abbreviations is given in Table I).

In the bright-field images, extinction, or optical density (OD), of algal objects was calculated by

$$\mathrm{OD} = -\lg\left(\frac{\mathrm{Transmission}_{\mathrm{measured}}}{\mathrm{Transmission}_{\mathrm{background}}}\right)$$

OD or (over the object) integrated OD (IOD) reflect the fading in visible light. Furthermore, for each object, means of chromaticity and hue were analysed.

For each of the spectral fluorescence channels gathered, containing the information of FLU1-3, the measured fluorescence intensity (MFIx) was transformed similar to OD by

$$\mathrm{TFIx} = -\lg\left(\frac{255 - \mathrm{MFIx}_{\mathrm{measured}}}{255 - \mathrm{MFI}_{\mathrm{background}}}\right)$$

(TFIx: transformed fluorescence intensity in spectral channel x),

where the background fluorescence was taken from the vicinity of the object. For each algal object, the mean of TFIx of all pixels was calculated and used as FLU1-3. To consider inhomogeneous distributions of the chloroplasts containing photosynthetic pigments, an automatic threshold was applied, which segments sub-regions inside the algal objects. For this purpose, RATS, a *r*obust *a*utomatic *t*hreshold *s*election (Kittler *et al.*, 1985) was calculated to detect the concentrated fluorescence inside the algal mask. The mean fluorescence intensity in these segments was calculated as FLU1*−FLU3*.

Fluorescence fading caused by repeated light exposition (“repeated excitation experiment”)

Phytoplankton was prepared as described. After sedimentation, the samples were immediately processed under the microscope. The image acquisition software was slightly changed: instead of the scanning pathway, 10 positions on the plankton chamber were manually selected with regard to interesting algal species. The microscope moved to these positions repeatedly (40 to 60 times=runs) in turn and acquired bright-field and fluorescence images as described. Thus, for each position, time series of bright-field and fluorescence images were obtained.

Segmented algae of different classes were selected by chance for feature analysis, excluding cells with FLU1 fluorescence near the background (FLU1 $>$ 0.3) to eliminate, for example, dying or dead individuals without chlorophyll. According to their presence in the samples, 40 Bacillariophyceae, 20 Chlorophyceae (together with Euglenophyceae, which have similar photosynthetic pigments), 9 Cryptophyceae, 7 Chrysophyceae, 7 Cyanophyceae and 4 Dinophyceae cells were gathered as examples for different algae classes. Time trends of fluorescence fading were analysed for each cell using Proc NLIN, SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Effect of sample storage on fluorescence (“storage experiment”)

Five hundred milliliters of phytoplankton water were taken from the pond and mixed. From this, four plankton chambers (PC1, PC2, PC3, PC4) were prepared as described according to the Utermoehl method. Subsequently,

1. PC1 was immediately processed under the microscope,
2. PC2 was stored for 12 days at room temperature (about 20°C) in darkness before processing under the microscope,
3. PC3 was stored for 12 days at room temperature and normal light (i.e. near a window, but without direct sunlight under light conditions of Germany in January) before processing under the microscope and
4. PC4 was stored for 12 days in a cool box (4°C) in darkness before processing under the microscope.

The cover slips of the plankton chambers were sealed with grease (Glisse al, Borer Chemie, Zuchwil, Switzerland) to avoid evaporation. This sealing was not perfect, so that bubbles occurred after 12 days of storage. However, as the whole preparation procedure was not anaerobic and contact with air reflects the normal situation during storing of plankton chambers, this was not regarded as serious. In the segmented images, algae of different classes were randomly selected for fluorescence analysis. Analyses of variance including Duncan tests were performed using Proc GLM, SAS 9.1, because of unequal numbers of observations in cells.

Development and testing of a classification procedure

The information gained from the repeated excitation experiment and the storage experiment, together with measured fluorescence intensities of randomly chosen non-algal objects in these experiments, were used to derive a linear hierarchical classification scheme, which discriminates algae with and without PE and non-algal objects. As water samples taken at different dates were used, a broad spectrum of algae and non-algal objects were included. In a first step, fluorescence of Chl *a* containing objects (algae) and non-algal objects and that of algae with and without PE were compared. Based on these results, the classification scheme was developed.

This procedure was subsequently tested by comparing the results of the classification scheme with a manual identification by an expert with long experience in phytoplankton analysis. For this evaluation, 13 samples were taken from different sides of the pond during a period of 16 weeks (from summer to late autumn). They were part of a study with weekly sampling and selected previously with reference to broad ranges algal density and dominating algae. The samples were taken in the same pond, but independently from those used to develop the classification scheme, e.g. on different sampling dates, day times and (partly) locations, and thus represent different algal assemblages. Owing to the long observation period, the study extended over the life spans of two fluorescence excitation lamps so that aging effects of the lamps were included.

Samples were processed without manual control of segmentation. Between 200 and 300 locations per sample were automatically chosen, following a spiral scanning pathway. Between fixation and image acquisition, the samples were stored for a few days in a dark cool box (4°C).

The images contained 1036–4704 objects per sample, of which 182–2533 were algae, belonging to a wide spectrum of species including all important freshwater algal classes and all size ranges usually found. Samples dominated by Bacillariophyceae, Dinophyceae or Chlorophyceae were included as well as samples without a clearly dominating group. The number of PE-containing Cryptophyceae ranged from 6 to 299 individuals per sample. Almost all PE-containing Cyanophyceae (>98%) belonged to Oscillatoriaceae, a group forming filamentous colonies (3–345 colonies per sample).

All objects were separated by the classification scheme into algae and non-algal objects as well as into algae with and without PE. Additionally, they were manually identified by the expert according to the taxonomic literature (Ettl *et al.*, 1978). The results of the manual identification, called "true" results, served as a reference for the test of the classification scheme; for each of the objects, the identification result of the classification scheme was qualified as true positive, true negative, false positive and false negative with respect to the expert identification. The overall performance of the classification scheme was estimated calculating sensitivity and specificity:

$$\text{Sensitivity} = \frac{\text{TP}}{\text{TP} + \text{FN}}$$

$$\text{Specificity} = \frac{\text{TN}}{\text{TN} + \text{FP}}$$

where TP is the number of true positives, TN the true negatives, FP the false positives and FN the false negatives.

RESULTS

Fluorescence fading in the repeated excitation experiment

As an example, the behaviour of a *Euglena* cell is shown in Fig. 1. In the bright-field image sequence, the colour of the cell became paler and shifted from green to reddish during the first 20–30 runs (Fig. 1a). Decreases in IOD, chromaticity and hue are shown in Fig. 1b. Fluorescence obtained by the I3 filter combination changed from red via orange and yellow to green. Interestingly, the total fluorescence intensity had an intermediate maximum around run 20 after the initial decrease. Here, the cells fluoresce green/yellow (partly slightly orange). This maximum was also visible in the red fluorescence obtained by filter N3, whereas almost no fluorescence emission appeared in the first and last runs for N3. Comparing fluorescence intensity courses of different algal species revealed similar results. Fig. 2

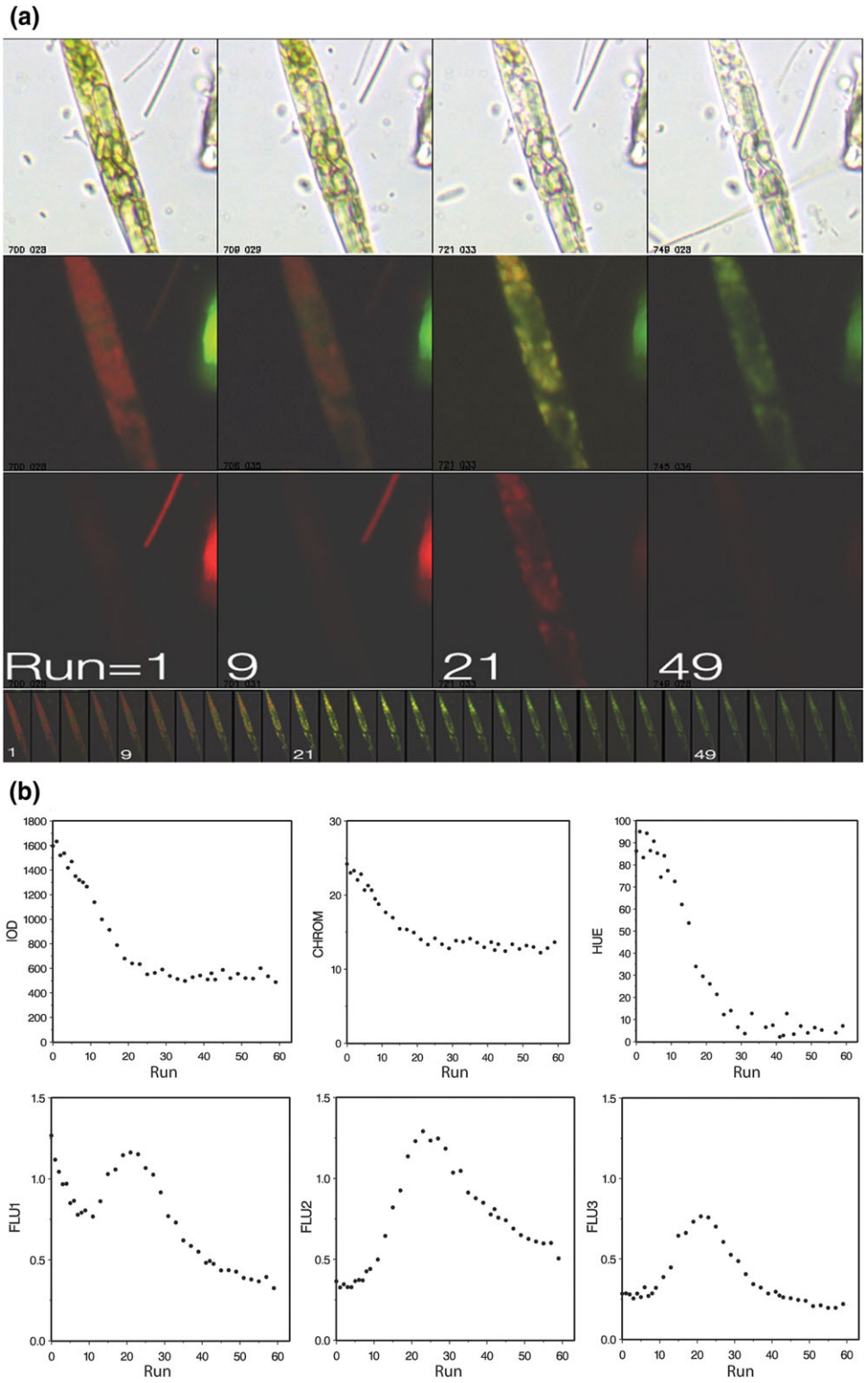

Fig. 1. Changes of a *Euglena* cell, caused by repeated exposure to fluorescence excitation light. (**a**) Bright-field images (first row), fluorescence images in filter system I3 (second row), and in filter system N3 (third row) after 0, 9, 21 and 49 runs. The lowest row shows every second run in filter system I3. (**b**) IOD [in arbitrary units (=a.u.)], chromaticity (CHROM, a.u.), hue (HUE, in degrees), FLU1, FLU2 and FLU3 (all a.u.).

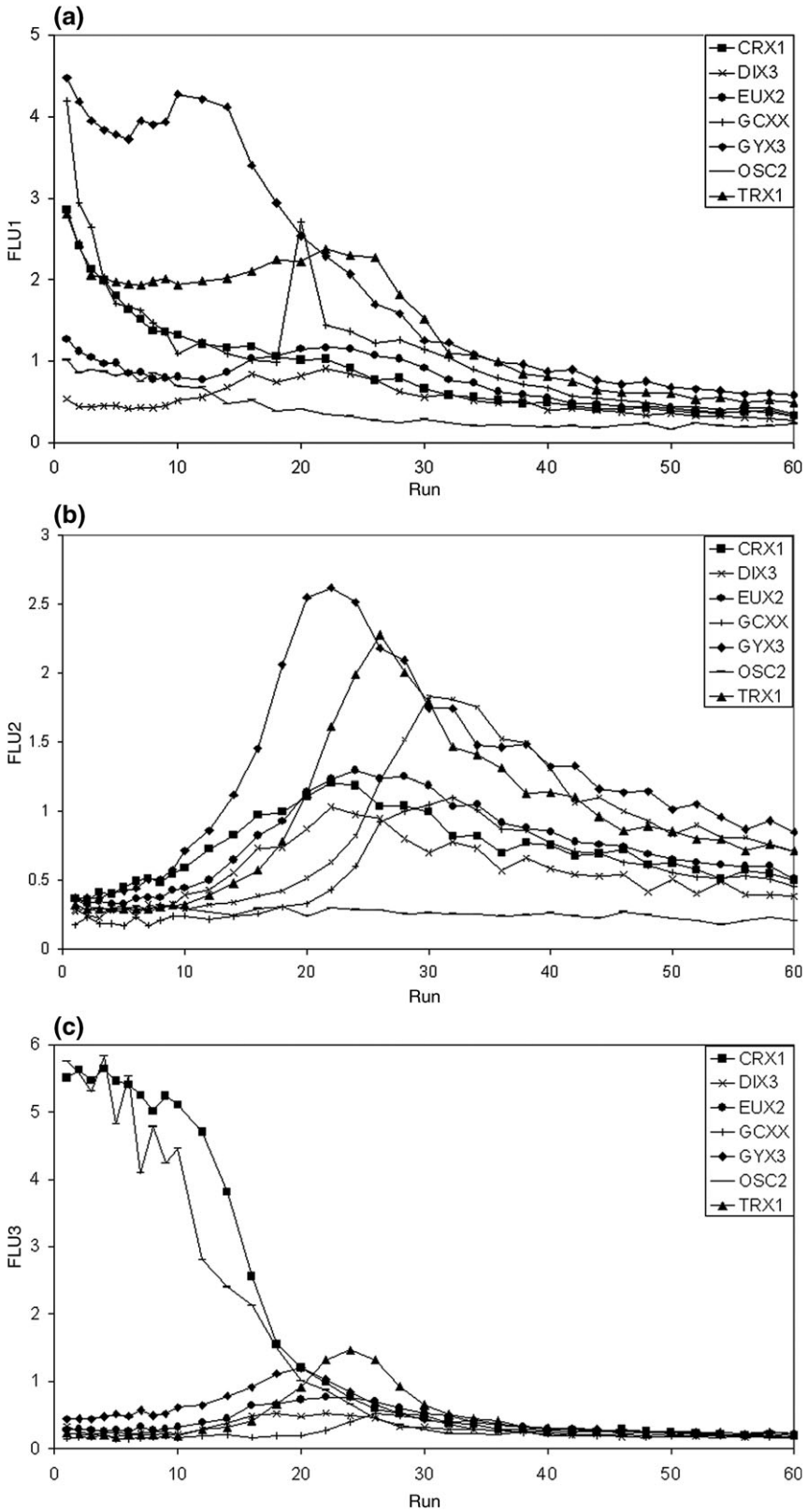

Fig. 2. FLU1 (**a**), FLU2 (**b**) and FLU3 (**c**) of different algal cells during the repeated excitation experiment. CRX1 is a *Cryptomonas* (class Cryptophyceae) cell, DIX3 a *Dinobryon* (Chrysophyceae), EUX2 a *Euglena* (Euglenophyceae), GCXX a Chlorococcales (Chlorophyceae), GYX3 a *Gymnodinium* (Dinophyceae), OSC2 an *Oscillatoria* (Cyanophyceae) and TRX1 a *Trachelomonas* (Euglenophyceae). All values in a.u.

shows the results for several species of different algal classes. Generally, FLU1 decreased during the first runs (Fig. 2a). Only for the *Dinobryon* cell, with very low starting FLU1-3 values, was this decrease almost absent. After 5–15 runs, FLU1 in most cells started to increase or showed at least a plateau (*Cryptomonas*). After a cell-specific maximum between runs 10 and 22, FLU1 decreased again. Only the *Oscillatoria* cell did not exhibit an intermediate maximum or plateau, whereas other *Oscillatoria* cells did (data not shown). These trends were found for all cells of the experiments. Almost all increased to an intermediate maximum/plateau, which was difficult to see for some cells with low start fluorescence. Size and time (run) of the maximum varied, with some consistency for cells of the same species. In the same filter combination, the green fluorescence (FLU2) was almost absent at the beginning, but subsequently increased to a maximum between runs 20 and 30, followed by a decrease (Fig. 2b). Again, only *Oscillatoria* showed no maximum (but other *Oscillatoria* cells did).

Two groups of cells can be distinguished by the red fluorescence obtained by filter N3 (FLU3), which was considered to measure PE fluorescence (Fig. 2c).

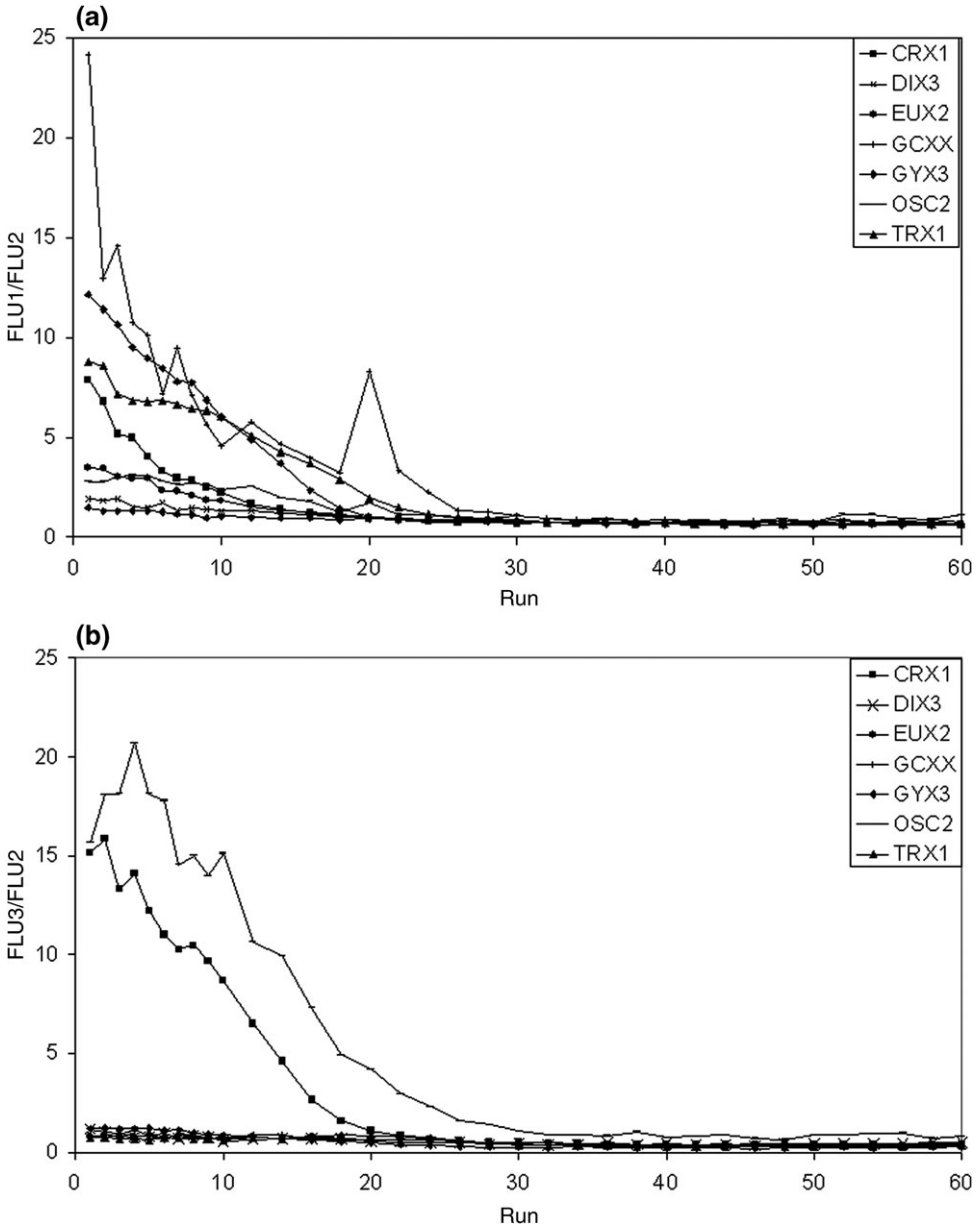

Fig. 3. FLU1/FLU2 (**a**) and FLU3/FLU2 (**b**) ratios of different algal cells during the repeated excitation experiment. Same cells as in Fig. 2.

Table II: Fluorescence ratio a at the first run and decrease rate b for the ratios FLU1/FLU2 and FLU3/FLU2 (best fit and lower–upper 95% confidence interval) and number of runs until the used thresholds were reached (mean and minimum–maximum)

	FLU1/FLU2				FLU3/FLU2		
	a	*b**100	Number of runs to 1.00	Number of runs to 0.85	*a*	*b**100	Number of runs to 2.00
Bacillariophyceae	4.5 (3.7–5.3)	6.5 (5.8–7.1)	23 (20–27)	26 (22–30)	1.0 (1.0–1.0)	—	—
Chlorophyceae	6.2 (3.0–9.4)	8.4 (5.4–11.4)	22 (13–34)	24 (15–37)	1.1 (0.8–1.4)	—	—
Chrysophyceae	1.8 (1.4–2.2)	2.4 (1.6–3.2)	24 (15–38)	31 (20–47)	1.1 (1.1–1.1)	—	—
Cryptophyceae	5.1 (4.2–6.0)	10.4 (8.8–12.0)	16 (13–19)	17 (15–21)	12.2 (9.4–15.0)	7.1 (5.2–9.0)	25 (19–34)
Cyanophyceae	3.7 (2.0–5.5)	2.1 (1.1–3.2)	63 (32–118)	70 (38–130)	8.8 (3.1–14.5)	2.7 (0.7–4.0)	55 (24–108)
Dinophyceae	10.8 (8.6–13.0)	7.6 (4.9–10.3)	31 (23–44)	33 (25–47)	1.1 (1.0–1.3)	—	—
Euglenophyceae	6.0 (4.4–7.7)	7.7 (5.2–10.1)	23 (17–33)	26 (19–36)	0.8 (0.7–0–9)	—	—

Cryptomonas and most *Oscillatoria* cells fluoresced strongly in the first run, which decreased after some delay. As fluorescence intensity was saturated in the first images, the delay was probably caused by the inability of the system to resolve the fluorescence decrease, rather than by the absence of it. In contrast, very low starting values for FLU3 of the other species (and some of the *Oscillatoria* cells) were found, which increased to intermediate peaks. The peaks occurred at similar runs as for FLU2. In cells with high initial FLU3 values no clear maxima were visible, which may be explained by a coincidence of the peak with the steep decrease of the initial fluorescence.

In summary, courses of FLU1 and FLU3, meant to represent Chl *a* and PE, seemed to be composed of two processes: one with an exponential decrease (in cells containing the pigments) and a second starting from zero (resp., background) to an intermediate maximum, followed by a decrease back to background values. Only this second curve was also present in FLU2. Obviously, the first process represents Chl *a* (FLU1) and PE (FLU3) bleaching. The second process represents the same unspecific fluorescence as FLU2 and is probably caused by the same substance (group). Curves of chromaticity and hue values of the bright-field image decreased during the first runs, followed by almost constant values. Generally, hues approached minima at about the same runs as FLU2 values reached their maxima, whereas chromaticity minima were reached a few runs earlier. As cell colour is dominated in most algal cells by photosynthetic pigments, this indicates a connection between FLU2 (and the intermediate maxima in FLU1 and FLU3) and those pigments.

This unspecific part of FLU1 and FLU3 affected identification of Chl *a* and PE and the investigation of their fading. As it also occurred in cells without the respective pigments (e.g. in algae without PE), it should be corrected as far as possible by FLU2. Tests using differences and ratios of the FLU values, as well as with untransformed fluorescence intensities (i.e. without logarithmic transformation) showed that best corrections were obtained by calculating the ratios of FLU1/FLU2 and FLU3/FLU2, respectively. Examples of results are shown in Fig. 3a and b. The intermediate maxima were almost or completely compensated for in most cells. Only in the green algae of the same species as GCXX did a short-term maximum remain.

The ratio furthermore enabled an optimal discrimination between algae and non-algal objects (see below). In images of some PE-containing cells, in which fluorescence FLU3 is saturated, FLU3/FLU2 sometimes showed an increase during the first runs, caused by an earlier start of FLU2 decrease compared with the saturated FLU3. However, this does not severely affect algal identification.

To enable quantitative statements of fluorescence behaviour of different algae classes over time (run), we fitted the data to a function. Although not fully functionally explicable, best fit was achieved by an exponential decline

$$\mathrm{FLU}(t) = a^* \exp(-b^* t).$$

where a is an intercept, initial fluorescence ratio (FLU1/FLU2 or FLU3/FLU2), t the time (run), b the decrease rate and FLU(t) the fluorescence ratio at run t.).

The first 15 runs of each cell were used to calculate a and b, as during this time most cells display a clear decline, to minimize a confusion between fading and the unspecific intermediate increase processes. Results are shown in Table II. For FLU1/FLU2, the initial ratio a differed between the algal classes, with low values for Cyanophyceae (3.7) and Chrysophyceae (1.8), and high for Dinophyceae (10.8) species. The intercepts correlated at least generally with the size (thickness) of the cells (Cyanophyceae and Chrysophyceae cells belonged

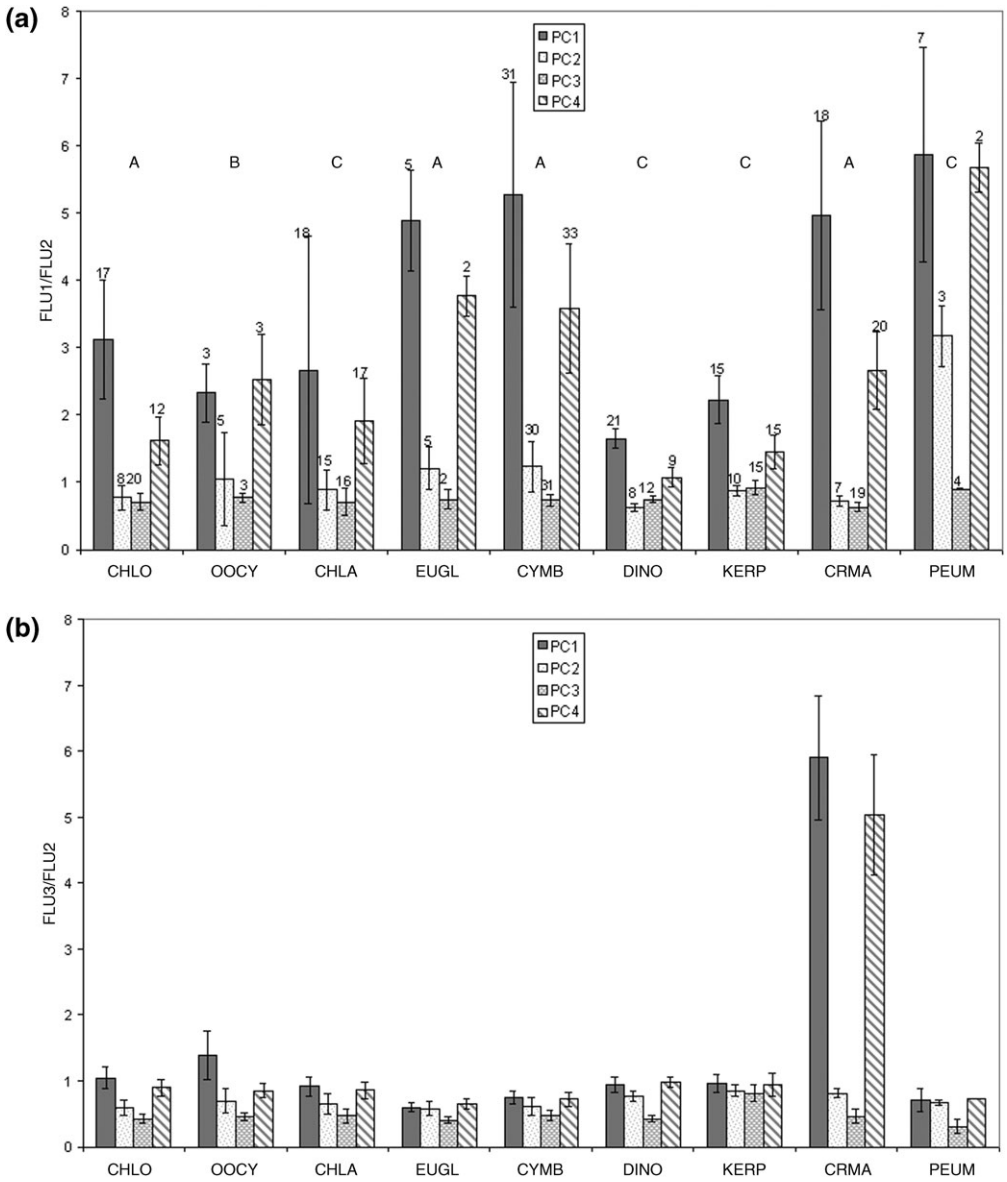

Fig. 4. FLU1/FLU2 (**a**) and FLU3/FLU2 (**b**) ratios in differently stored algae. PC1, no storage; PC2, darkness, room temperature; PC3, normal day and night, room temperature; PC4, darkness, cooled. Numbers of analysed cells are given in (a) above the columns. (A–C) in (a) indicate significance groups, i.e. groups for which the decline behaviour during the storage significantly differed (Duncan test, Proc GLM, SAS 9.1). CHLO, Chlorococcales; OOCY, *Oocystis*; CHLA, *Chlamydomonas* (all Chlorophyceae); EUGL, *Euglena* (Euglenophyceae); CYMB, *Cymbella* (Bacillariophyceae); DINO, *Dinobryon*; KERP, *Kerphyrion/Pseudokerphyrion* (both Chrysophyceae); CRMA, *Cryptomonas* (Cryptophyceae); PEUM, Peridiniales (Dinophyceae). Investigated cells of CHLO (round cells with 15–20 μm diameter) and perhaps PEUM belonged to various species, the other to a single each.

to the smallest, Dinophyceae to the largest). FLU1 indicates the amount of fluorescing Chl *a* pigments normalized per area, thus thick cells tend to give higher initial FLU1 values. In contrast to FLU1, initial values of FLU2 showed no correlation with the cell thickness (data not shown). The decrease rate *b* was associated with *a*, i.e. cells with high starting values show a faster decline. For most classes, FLU1/FLU2 thresholds were reached on average after similar runs (between 22 and 31 runs for threshold 1.00 and between 24 and 33 runs for threshold 0.85). Cryptophyceae reached the thresholds slightly earlier (after 16 and 17 runs), whereas in Cyanophyceae clearly later (after 63 and 70 runs). The Chlorophyceae showed some variation, but, regarding the lower 95% confidence interval, most cells need at least 13 (threshold 1.00) or 15 (threshold 0.85) runs to reach the threshold, thus these values may be considered "safe". Regarding the fading to the

thresholds, some cells of Chloro-, Chryso- and Cryptophyceae were most critical.

As expected, only Cyano- and Cryptophyceae have high FLU3/FLU2 starting values *a* (12.2 and 8.8, respectively). Decrease rate *b* was calculated as 7.1 and 2.7. Again, the values for the thicker cells (Cryptophyceae) were higher than that of the thinner ones (Cyanophyceae). The other classes provided only background ratios around 1.0. Threshold 2.00 was reached after 25 and 55 runs, the lowest lower 95% confidence limit was 19 runs. Owing to the FLU3 saturation during the first runs, the real decrease rates were higher. However, as the real starting values are also underestimated, threshold runs should be only moderately incorrect.

It has to be mentioned that *a* and *b* were calculated using only the first 15 runs for each cell, i.e. values for later runs were extrapolated. However, the "safe" limits of 13 or 15 runs lie within the calculated range.

Fluorescence fading during sample storage

Fading during water storage was analysed in eight different algal groups, belonging to different classes (Fig. 4). As the ratio of FLU1/FLU2 and FLU3/FLU2 has turned out to be adequate for discrimination purposes, rather than FLU1 or FLU3 itself, we will primarily present results based on these ratios.

Compared with cells processed without storage (PC1), in cells stored 12 days at 4°C in darkness (PC4) FLU1/FLU2 had declined by a mean of 31% (maximum 48% in Chloroccoccales) in most algal groups (Fig. 4a). Unusually, it had increased in *Oocystis* (however, the number of investigated cells was low). Storage at room temperature in darkness (PC2) increased fading considerably (46–85%, mean 67%) in all groups. Exposure to light (PC3) had only a small, unambiguous additional effect (55–87% fading, mean 75%). A Duncan two-way ANOVA was calculated to investigate the FLU1/FLU2 reduction in PC2, PC3 and PC4 compared with PC1 (in percent) for the different species. All storage methods showed significant differences to PC1, but also between each other. The test confirmed the order of decrease (PC4 < PC2 < PC3).

Regarding the species, the test showed three significantly distinct groups (Fig. 4a). The order of reduction was group A<B<C. This grouping points to a possible relevance of different pigment compositions in different algal classes. The carotinoid rich *Dinobryon*, *Kerphyrion/Pseudokerphyrion* and Peridiniales (but not the *Cymbella*) species were in group C, whereas most species in group A are dominated by chlorophyll. However, this did not fully explain the grouping. Similar to the repeated excitation experiment, ratios in many small cells with low starting values in PC1 (*Oocystis*, *Dinobryon*, *Kerphyrion/Pseudokerphyrion*, *Chlamydomonas)* tended to be less affected by decrease of FLU1/FLU2 than the larger and more fluorescing cells of *Euglena*, *Cryptomonas* or *Cymbella*.

FLU3/FLU2 in Cryptophyceae (no Cyanophyceae found) was also almost preserved after storage in darkness at 4°C (15% decrease), whereas it decreased in PC2 and PC3 by 86–92% (Fig. 4b). FLU3/FLU2 intensity in the other algal groups, which did not contain PE, was lower and showed only small changes after storage. The differences between Cryptophyceae cells in PC1 and PC4 as well as between cells in PC2 and PC3 did not reach significance, whereas they did comparing cells of both respective groups combined according to one-way ANOVA.

FLU1 and FLU3 showed similar changes as did their ratios with FLU2, although the decrease after storage compared with PC1 was slightly lower (data not shown). In contrast, FLU2 generally increased. Whereas this increase for PC4 was low (mean 10%) and did not occur in all algal groups, FLU2 in PC2 and PC3 mostly reached values between 150 and 250% (but up to 500%) of those in PC1.

Development and testing of a classification procedure

Separation of algae/non-algal objects

To enable a separation of algae and non-algal objects by Chl *a* fluorescence, we first compared fluorescence emissions of both. FLU1 of algae [taken from the repeated excitation experiment, $n = 87$] was 1.15 ± 0.92 SD. Especially some small algae of the Chrysophyceae (0.36 ± 0.10 SD, $n = 7$) and Cyanophyceae (0.76 ± 0.47 SD, $n = 7$) showed low FLU1 intensities.

The analysed non-(living-)algal objects comprised a wide spectrum of objects, e.g. empty shells of algae, cell fragments, zooplankton, inorganic pieces and objects of unknown origin. FLU1 varied between 0.02 and 0.71, mean 0.29 ± 0.19 SD ($n = 24$). A repeated exposure to excitation light [as decribed in the repeated excitation experiment] resulted in a fading of most objects ($n = 13$) with a rather irregular time course, whereas 10 objects showed no clear tendencies. In contrast, FLU1 of the object with lowest value increased. After 42 runs, FLU1 had reached values between 0.14 and 0.26 (mean 0.19 ± 0.04 SD).

FLU2 of non-algal objects varied between 0.11 and 1.27 (mean 0.45 ± 0.46 SD). After 42 runs, values were 0.14–0.52 (mean 0.23 ± 0.11 SD). Some of the objects

(diatom frustules) showed an increase during the repeated excitation experiment from very low starting values (around 0.12), but the time course for these non-algal objects was rather indifferent.

The results indicate the impossibility of an unambiguous separation of algae and non-algal objects using a simple threshold of the chlorophyll fluorescence intensity (represented by FLU1), as an overlapping range mainly between intensities of 0.35 and 0.70 occurs.

In non-algal objects, FLU1 represents an unspecific fluorescence produced by different "background" substances. Generally, this background fluorescence is not as restricted as pigment fluorescence to specific bands in the excitation and emission spectrum, but usually emits as well in the FLU1 channel as in others, for example, in FLU2. An increased FLU1, caused by these substances, is usually connected with an increased FLU2, whereas chlorophyll emits almost only in FLU1. Therefore, FLU2 can be used to separate chlorophyll containing objects from others.

A correction of FLU1 by the FLU1/FLU2 ratio was more suitable to separate algae from non-algal objects than other transformations, e.g. differences between FLU1 and FLU2 (data not shown). Furthermore, variance between cells of one algal species or one class was lower, especially for algae with low Chl *a* fluorescence. This supports the use of the ratio proposed in the repeated excitation experiment.

The FLU1/FLU2 ratios of 224 algae of different classes and 24 non-algal objects, acquired from samples of different natural freshwaters at different times, were analysed to investigate the separation potential of the ratio. The algae comprised large, brightly fluorescing algae as well as small algae with small chloroplasts. Some algae had dark shells (*Trachelomonas*, *Kerphyrion/Pseudokerphyrion*), which may absorb parts of the excitation and fluorescence.

The FLU1/FLU2 ratio ranged from 0.71 to 24.15 (mean 4.38 $\pm$ 2.89 SD) for algae and from 0.12 to 2.05 (mean 0.86 $\pm$ 0.43 SD) for non-algal objects. An overlap occurred mainly for small algae with low FLU1 ($<$0.4) and large non-algal objects with high FLU1 ($>$0.4). This overlap suggested a separation using different FLU1/FLU2 thresholds. Objects with very low FLU1 ($<$0.15) were classified as non-algal objects. Separation was difficult for objects with FLU1 between 0.15 and 0.4. Especially for algae, in which fluorescing chloroplasts only fill a small part of the object (e.g. certain *Navicula*, *Dinobryon*), the mean overall FLU1 was low and sometimes governed by unspecific fluorescence. For these, the use of FLU1* and FLU2* was more adequate, as these features focus on the most fluorescing structures in the objects, in the case of (non-cyanobacteria) algae mainly on chloroplasts. Best results were obtained with a FLU1*/FLU2* threshold of 1.0. Applying this threshold, all non-algal objects and all algae except one were correctly classified. The misclassified algal cell was a small *Cymbella* with a deformed, shrunken chloroplast, probably an inactive, dying cell. Some other algae and non-algal objects were close to the threshold, indicating a risk of misclassification. For a more reliable avoidance of misclassified algae or non-algal objects, threshold could be decreased or increased. For objects with FLU1$>$0.4, a threshold of 0.85 gave satisfactory results. As for this group, no algae with a FLU1/*FLU2* ratio $<$0.91, and no non-algal objects with $>$0.76 were found, the proposed threshold is reasonably safe. With the exception of the described *Cymbella* cell, all objects were classified correctly by these thresholds.

Separation of algae with/without PE

We analysed the potential of PE fluorescence (in FLU3) to discriminate algae with and without PE. FLU3 of all investigated Cryptophyceae (n = 27) ranged from 0.98 to 2.35 (mean 2.01 $\pm$ 0.28 SD), of Cyanophyceae from 0.13 to 2.35 (mean 1.25 $\pm$ 1.00 SD, n = 7) and from 0.09 to 1.57 for all other algae investigated (mean 0.27 $\pm$ 0.19 SD, n = 188). For the fading course in algal cells, see the results of the repeated excitation experiment. FLU3 of non-algal objects started with values

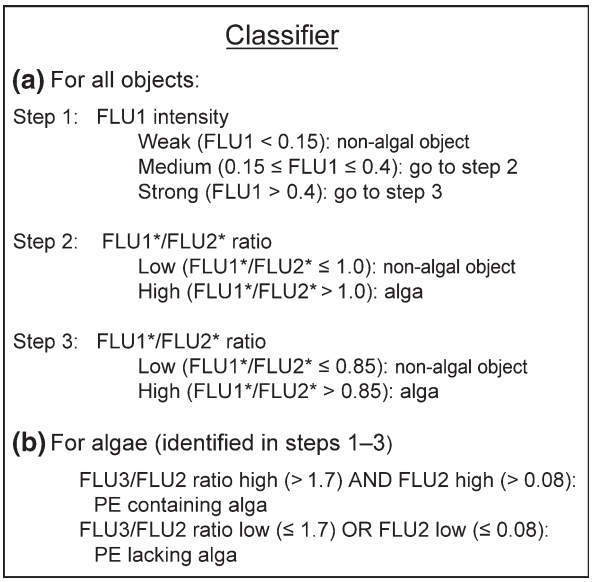

Fig. 5. Classifier, based on fluorescence information, for objects identified by image analysis. (**a**) First, all objects are divided into algae and non-algal objects by a three-step procedure. (**b**) Subsequently, objects identified as algae are further divided into PE-containing and -lacking algae. For more information, see the text.

Table III: Sensitivity (positive correctness) and specificity (negative correctness) of algae classifiers 1, 2 and 2a (calculated with respect to all objects in the respective sample) and the classifier for PE-containing algae (calculated with respect to all algae), analysed in 13 phytoplankton samples

				Classifier 1		Classifier 2		Classifier 2a	PE classifier	
Sample	Total number of objects	Number of algae	Number of PE-containing Cryptophyceae	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Sensitivity	Specificity
1	1 244	316	15	0.73	1.00	0.93	0.99	0.98	1.00	0.90
2	2 665	742	299	0.77	1.00	0.94	0.99	1.00	0.99	0.99
3	4 704	1 981	151	0.95	0.97	0.98	0.93	1.00	0.99	0.99
4	2 955	882	22	0.91	0.99	0.97	0.99	1.00	1.00	1.00
5	2 270	454	17	0.73	0.99	0.93	0.95	0.94	1.00	0.98
6	2 565	425	60	0.62	1.00	0.88	0.98	0.90	1.00	1.00
7	1 733	782	20	0.97	1.00	0.99	0.99	1.00	1.00	0.99
8	1 946	182	50	0.83	0.99	0.98	0.93	0.99	0.94	0.97
9	2 444	604	67	0.94	1.00	0.98	0.98	0.99	1.00	1.00
10	4 650	2 533	83	0.83	0.98	0.90	0.94	1.00	0.93	1.00
11	2 570	1 101	213	0.83	0.99	0.90	0.95	1.00	0.92	0.99
12	2 058	312	27	0.59	0.99	0.83	0.97	0.92	1.00	0.98
13	1 036	388	6	0.95	0.99	0.99	0.98	0.99	1.00	0.97
Total	32 840	10 702	1030	0.85	0.99	0.94	0.96	0.99	0.97	0.99

Specificity of classifier 2a is identical to that of classifier 2.

between 0.11 and 1.20 (mean 0.35 ± 0.34 SD) in the repeated excitation experiment. Especially the objects with higher intensities faded following a course best described by an exponential function. After 42 runs, FLU3 ranged from 0.15 to 0.25 (mean 0.18 ± 0.03 SD).

Thus, overlapping was again found, e.g. between small Cyanophyceae (with low FLU3) and large Chlorophyceae. Identification of weakly fluorescing Cyanophyceae could not be improved by calculating FLU3* and FLU2*, as usually the whole cells, which do not contain chloroplasts, fluoresced.

To improve separation, FLU3/FLU2 ratios were calculated, analogous to the FLU1/FLU2 ratio. The ratios ranged from 4.36 to 15.22 (mean 7.75 ± 3.05 SD) for Cryptophyceae, 1.21 to 15.67 (6.56 ± 5.21 SD) for Cyanophyceae and 0.36 to 2.52 (0.93 ± 0.23 SD) for other algae. Cyanophyceae showed a clear dichotomy: three individuals with values between 1.21 and 1.63 probably lacked PE, whereas the values of the other (6.82–15.67) indicated PE presence. A separation of PE-containing Cryptophyceae and other algae was unambiguously possible by setting a threshold for FLU3/FLU2 somewhere between 3.0 and 4.0.

Small algae (and non-algal) objects with very low FLU2 tend to have a degree of FLU3 background fluorescence, resulting partially in high FLU3/FLU2 ratios. Thus, additionally an FLU2 threshold (FLU2$>$0.08) is needed for identification of PE-containing algae.

Classification scheme

Based on these results, a classification scheme was developed to separate algae from non-algal objects and PE-containing algae from PE-lacking algae (Fig. 5). It was designed to achieve a classification optimum with low computational effort.

Testing the classification scheme

Finally, we tested the quality of the derived classification steps.

Separation of algae from non-algal objects. Specificity is the proportion of non-algal objects identified as non-algae. It was near 1.00 for the classification scheme used (classifier 1 in Table III). However, sensitivity, which indicates the proportion of all algae identified as algae, was lower (0.59–0.97). Whereas almost all objects identified as algae were true algae, a considerable number of algae were classified as false negatives. Most of them were algae with low FLU1 ($<$0.15), including algae with small chloroplasts (compared with the body size), algae with light-absorbing shells, insufficiently fixed algae, dying algae or those with a low physiological state. Therefore, classifier 1 was slightly modified, i.e. objects with weak FLU1 (step A1 in Fig. 5) were further divided in algae (with ratio FLU1/FLU2$>$1.4) and non-algal (ratio $\leq$1.4). This classifier 2 lead to a slight reduction of specificity (0.83–0.99), whereas sensitivity improved (0.93–0.99). False-negative algae were mainly

thin (<2 μm) and long (Oscillatoriaceae), some with small chloroplasts (*Synedra*) or were difficult to focus (*Ankistrodesmus*). Especially, the Oscillatoriaceae caused problems. Being thin, naturally pale and without organelles, it was often difficult to decide, whether a colony was dead. *Oscillatoria* colonies often live in dense mats at surfaces. Thus, some floating filaments were probably dead or dying. Deleting this group (classifier 2a) further improved the sensitivity (0.90–1.00). Without *Synedra* and *Ankistrodesmus*, sensitivity was 0.98–1.00 (data not shown). Other false negatively identified cells belonged to species with strongly coloured shells (e.g. *Trachelomonas*, *Pseudokerphyrion/Kerphyrion*).

Most false-positive objects were very small particles (around 5 μm diameter), which occurred in some samples in high densities. Some of them fluoresced in a way similar to Chl *a* containing algae. Possibly they originated from algae disintegrated e.g. by the fixation or plant material with residues of Chl *a*. As these objects were of non-algal-like, rather unspecific shape and colour, they should be identified by means of some morphological or colourimetric features. The remains were small objects of uncertain origin, possibly destroyed algae cells.

Separation of PE-containing from PE-lacking algae. Some, but not all Oscillatoriaceae species were known to contain PE. Here, between 0 and 88% of the Oscillatoriaceae colonies identified as algae (65% of all colonies) were classified as PE-containing. However, as Oscillatoriaceae species are difficult to identify, literature information of PE content is not available for all species, and due to the problems of identifying living colonies, they were not evaluated in detail. Other Cyanophyceae, which may also contain PE, were very few in numbers in our samples and therefore neglected in this analysis. The remaining PE-containing species belong to the Cryptophyceae. For these, step (b) of the classifier (Fig. 5) showed high sensitivity (between 0.92 and 1.00) and specificity (between 0.97 and 1.00). Fifteen of 16 Cryptophyceae individuals, which were identified as false negatives in the sample with the worst sensitivity (sample 11), were located in the centre of the plankton chamber. In this area, the spiral acquisition pathway may result in an overlap of excitation regions and thus in an increased bleaching by multiple excitations. Therefore, changing the pathway could further improve the result. FLU3/FLU2 ratios of a few Dinophyceae species overlapped with those of PE-containing algae, decreasing the specificity. Apart from these species, specificity was 0.98 or higher. The remaining false-positive objects mainly consisted of protozoa which may contain PE-containing algae (as food or symbionts). However, identification of Dinophyceae and zooplankton could easily be made by introducing additional features (e.g. morphometric) in the image analysis.

DISCUSSION

Autofluorescence signatures provide relevant information for taxonomic identification of algae and, thus, are used in different identification methods (Sieracki *et al.*, 1990; MacIsaac and Stockner, 1993; Schäfer *et al.*, 1995; Babichenko *et al.*, 1999; Jochem, 2001; Poryvkina *et al.*, 2001; Jacquet *et al.*, 2002; Bodemer, 2004; Rodenacker *et al.*, 2006). However, only few studies applied it in the analysis of microscopic images (Sieracki *et al.*, 1985; Nagata *et al.*, 1994; Walker *et al.*, 2002; Belykh *et al.*, 2006; Ernst *et al.*, 2006). If detailed taxonomic species information in natural samples is required, microscopic evaluation remains the method of choice (Rutten *et al.*, 2005). Generally, sophisticated microscopic technologies such as CLSM or spectral analysis can lead to good results (Larson and Passy, 2005). However, such elaborate technologies are expensive and not always available. We thus investigated the potential and properties of algal autofluorescence in a normal bright-field and fluorescence microscope with computational analysis coupled with an image acquisition and analysis system.

Fluorescence fading behaviour

The fluorescence measured as FLU1 from the I3 filter system does not represent pure Chl *a* autofluorescence. We first assumed that the unexpected intermediate FLU1 peak in the fading experiment is explained by the presence of non-Chl *a* pigments in the cell cytoplasm or wall, which shadow Chl *a*. Decay of these pigments could be induced by the excitation light and lead to an increase of Chl *a* excitation and thus fluorescence in this spectral range. However, according to a mathematical model, such pigments could delay fading of Chl *a*, but not explain an intermediate maximum (J. Müller, Garching/Munich, Germany, personal communication).

After fixation with paraformaldehyde, a part of Chl *a* was reported to be transformed to phaeopigments (Navaluna *et al.*, 1989). These Chl *a* degradation products emit autofluorescence, which partly overlaps with the Chl *a* autofluorescence. However, the peak is not related to the value of the initial Chl *a* fluorescence. Furthermore, Fig. 2a shows that the FLU1 increase to the peak started with a certain time delay compared with the initial fading. Thus, the intermediate

maximum does not present a Chl *a* degradation product, which is in accordance with the results of Tang and Dobbs (Tang and Dobbs, 2007).

The peak is also visible in FLU2 and FLU3, indicating rather broad excitation and emission spectra. Primarily from higher plants, an unspecific autofluorescence of mainly blue–green fluorescence (BGF) emission is known (Yentsch and Horan, 1989). A number of different substances, e.g. flavonoids, flavins (e.g. FAD), cinnamic acids (e.g. ferulic acid), betaxanthine and pyridine nucleotides [e.g. NAD(P)H] is responsible for BGF (Stober and Lichtenthaler, 1993; Morales *et al.*, 1994; Cournac *et al.*, 2002; Gandia-Herrero *et al.*, 2005; Lichtenthaler *et al.*, 2005). Some of them occur in the whole cell, and others primarily in specific compartments as cell wall (some cinnamic acids) and chloroplasts (NADPH, some flavins) (Latouche *et al.*, 2000; Hideg *et al.*, 2002). At least for some of these substances, emission spectra range into or have their maxima in the green/yellow/or even orange range (Chorvat *et al.*, 2005). Non-plant and -algal organisms also possess many of these substances [e.g. FAD, NAD(P)H] and thus BGF. This at least partly explains the measured FLU1 in non algal objects. BGF in algae is not well investigated, and the composition of the described (and other) BGF fluorescing substances in different algal species is widely unknown. Recently, Tang and Dobbs (Tang and Dobbs, 2007) reported that a green autofluorescence, which occurred in species of different algal classes and showed similar fading behaviour as FLU2, interfered with detection of green-fluorescing stains.

In leaves of higher plants, it was found that decreasing red Chl *a* fluorescence is accompanied by increasing BGF (Morales *et al.*, 1994). Similar results were found for algae by Tang and Dobbs (Tang and Dobbs, 2007). There are two reasons for this: (i) Chl *a* (and carotinoids) absorb the excitation light of BGF and (ii) especially Chl *a* reabsorbs the fluorescence emitted by BGF. This most probably also explains the fluorescence peaks in all channels in our experiment, including FLU1: the photodegradation of fluorescing Chl *a* pigments due to repeated excitation which decreases the suppression of BGF by this pigment. Subsequently, the BGF carrying pigments fade themselves. The connection between the decrease of chlorophyll red autofluorescence and the increase of "green autofluorescence" in algae (which at least partly is identical with BGF), as reported by Tang and Dobbs (Tang and Dobbs, 2007), supports our interpretation.

Almost all investigated algal cells in this study showed the intermediate fluorescence peak, although the time course of the peak and its height varied, sometimes resulting only in a FLU1 plateau. Tang and Dobbs (Tang and Dobbs, 2007) proposed an interplay of production of green fluorescing pigments, e.g. as degradation products of other substances after cell fixation, and their decomposition or leakage as an alternative explanation for the green fluorescence. However, the explanation seems unlikely, as such a scenario could hardly explain the time course of the unspecific fluorescence in our experiments, e.g. the varying delay phases before the start of the intermediate fluorescence increase. Furthermore, the existence of substances causing BGF, which is suppressed by emission and excitation blocking by Chl *a* and carotinoids, is known (Morales *et al*, 1994), whereas the relevant existence of BGF or green fluorescing substances, which were produced after cell fixation, and for which the production was promoted by repeated fluorescence excitation as found in our study, still has to be proven.

As the intermediate fluorescence peak is not limited to the blue/green emission range, we keep referring to it as unspecific fluorescence in the following (instead of "green fluorescence" or "BGF").

Note that in all cells of one unidentified Chlorococcales species, labelled GCXX in Fig. 2, an additional process seemed to overlay the course of FLU1. These cells are characterized by a sudden burst of fluorescence which subsequently decreased. It seems that a depot of a fluorescing substance (possibly Chl *a*) is released suddenly, perhaps by the destruction of a protection mechanism. This burst is not connected with BGF. It does not appear in FLU2 and thus cannot be corrected. However, it increases the FLU1/FLU2 ratio in algal cells and therefore it does not interfere with the correct identification of the cells as algae.

Intermediate peaks in algae without PE, probably caused by the same process, are also visible in FLU3. The lack of a clear peak in PE-containing cells is probably related to the strong decay of PE fluorescence, which masked simultaneous increases of unspecific fluorescence.

The same effects as found for fading, caused by repeated excitation, seemed to appear during algal storage. Decrease in FLU1 (and FLU3) is accompanied by an increase in FLU2. This process is promoted by high (room) temperature and/or light exposure during storage. Obviously, storage had the same effects on the composition of fluorescing pigments as fading due to exposure to excitation light. The spectral shift of autofluorescence emission from red to green during storage in algae that were fixed with 5% formaldehyde (Larson and Passy, 2005) can be interpreted analogously.

Principally, a restriction to small excitation and emission bands, optimized to discriminate Chl *a*

autofluorescence from BGF, appears desirable. However, limited epifluorescence filter sharpness and a decrease in measured fluorescence intensity using small bands restrict such improvements. Thus, discrimination of Chl *a* from unspecific fluorescence by a correction of FLU1 with FLU2 is more adequate. The applied correction procedure, division by FLU2, worked better than the other methods tested and achieved results sufficient for our purposes.

Chl *a* and PE fading due to repeated excitation

Derivation of a quantitative fading model to describe the loss of pigments is a difficult task. Besides technical problems of fluorescence pigment quantification, e.g. definition of cell boundaries in fluorescence images, influence of the focus and shielding in larger cells, a complex chemistry of the molecules in algae exists (Rowan, 1989; Larkum *et al.*, 2003).

The proportions of fluorescing Chl *a* and PE pigments in photosystems vary due to degeneration of the photosystem complex and decoupling of the electron chain during cell fixation, storage and fading. Other autofluorescing or non-autofluorescing substances, which themselves are affected by fading, interfere with the Chl *a* and PE autofluorescence, e.g. by shading, re-absorption of emitted light, energy transfer or other physico-chemical interactions. These effects change in an unpredictable way during fading, making the development of an exact, mechanism-based fading model impossible.

It cannot be explained unambiguously why larger cells tend to have higher decrease rates. Shielding effects, which should preferably occur in larger cells, would result in a delayed fading, i.e. lower *b* values. The simple exponential function is just a coarse approximation to the hardly understood physico-chemical processes ruling the decline of the FLU1/FLU2 resp FLU3/FLU2 ratios. However, the courses of the ratio observed followed the equation quite well, thus it is sufficient for the purposes of this study.

As shown by the numbers of runs to reach the thresholds, normally the fluorescence fading caused by repeated excitations is too slow to cause a misidentification of algae in fresh samples. Nevertheless, the above described problems with Cryptophyceae indicate that repeated excitations, e.g. by neighbouring scanning fields, should be avoided. To limit unintended excitation, the aperture of the fluorescence light should be closed as far as possible. Regarding the chosen thresholds for the ratios, our results suggest that some algal classes may be especially sensitive for fading (Chlorophyceae, Cryptophyceae). The PE fluorescence was less sensitive for fading than Chl *a*, indicating that, as long as algal identification by the FLU1/FLU2 ratios works, no problems concerning identification of PE-containing algae will arise.

Although investigation of the susceptibility of colour information for bleaching was not the focus of this study, some results of chromaticity and hue in the fading experiment indicated a similar behaviour of this feature. As both cellular properties originate from at least partly the same pigments, this is not surprising. Thus, the conclusions concerning information intensity decline in fluorescence also apply for colour.

Storage of samples

Storage of samples increases the problem of fluorescence information loss. A suitable, i.e. cold and dark storage is essential, as only 12 days at room temperature or in the light resulted in fluorescence values insufficient for classification.

Assuming an average decrease rate *b* of 0.06 for FLU1/FLU2 during the repeated runs in the fading experiment, a mean decrease of 31% during the 12 days cold and dark storage is equivalent to the loss of fluorescence information after about six runs. The fading experiment indicates that after 13–15 runs identification was difficult. Extrapolating this roughly to the storage experiment, the identification thresholds may be reached after about 4 weeks of storage in cold darkness, for Chlorophyceae ($b = 0.084$, 48% decrease during 12 days storage) even after about 3 weeks. Fluorescence information loss due to storage adds to other fading causes (e.g. due to repeated excitation), thus storage time should be reduced as far as possible.

Similar to the fading due to repeated excitation, fading rates during storage revealed differences between species. Taxonomic classification partly explained these differences, probably due to varying pigment compositions. However, an assignment of cells to algal classes by these differences was not possible, as it was superimposed by other factors such as cell size.

We tried to adapt the fixation procedure as a trade-off between fluorescence preservation, preservation of morphological information (e.g. shape) and an economical use of the toxic glutaraldehyde. However, if long-term preservation of fluorescence is important, an optimization of the fixation regarding this aspect may increase the possible storage times. Furthermore, the use of fading blockers such as citifluor may improve the situation. The influence of these substances on the features considered has to be investigated.

As for fading induced by repeated fluorescence excitation, PE fluorescence in Cryptomonaceae cells appeared to be less sensitive than Chl *a* fluorescence fading during storage.

The experiments on fading during repeated excitation and storage were based on a limited, although not too low number of cells and species. They were not selected for representativeness and thus can give only indications for other species or environmental conditions. The FLU1*/FLU2*, which was applied in the classification scheme, referred to regions of highest fluorescence intensities. The sizes of these automatically calculated regions varied, i.e. they decreased when the fluorescence intensity decreased. For reasons of comparability, we thus used FLU1/FLU2 in the storage as well as the repeated excitation experiments, which referred to the fluorescence in the whole cell. However, as FLU1*/FLU2* focused on regions containing Chl *a* (chloroplasts), their starting values were higher and the time until classification thresholds were reached was longer. Therefore, the provision for fading problems due to excitation light or storage can be regarded as conservative.

Chl *a* and PE classification

The quality of both Chl *a* and PE separation reached in this study is rather high. This especially holds true, as sensitivity and specificity were not calculated overall, but separately for all samples. These include samples with relatively low density of Chl *a* or PE-containing algae and high density of other particles, which often cause counting problems. The sampling location was a small pond, containing more particles than most pelagic zones of larger waters. Furthermore, samples of different seasonal times and phytoplankton compositions were considered. Differing test aims and designs make a direct comparison with other studies impossible. However, the sensitivity and specifity reached indicate that the procedure presented works well, despite the impeding factors (Godhe *et al.*, 2007). As usual, a trade-off between sensitivity and specificity has to be made regarding the test design. Varying the classification scheme, e.g. the thresholds, allows an adaptation to specific test goals. Depending on the scientific question, the thresholds in the classifier can be set higher (allowing less false positive, but more false-negative results) or lower (more false-positive, but less false-negative results).

Generally, two main sources of uncertainty complicate correct classification. On one hand, the algae show high variability. This is caused, for example, by differences between species, life stages, seasons, nutrients, light intensities and other environmental conditions (Jacquet *et al.*, 2002; Le Floc'h *et al.*, 2002). Our test considered a large part of the growth season with different nutrient and light conditions. However, it is possible that under extreme circumstances the thresholds could be inadequate. As discussed, photosynthetic pigments, e.g. some phycobilins, as well as other cellular substances show red fluorescence in the Chl *a* detecting filter system. Similar problems apply to the filter system detecting PEs, which present a group of different molecules with different fluorescence spectra (MacIsaac and Stockner, 1993, Poryvkina *et al.*, 2001; Larkum *et al.*, 2003). Some algal species, e.g. some Cyanophyceae, are known to contain low concentrations of Chl *a* (Yentsch and Phinney, 1985). PE are restricted to mainly Cyano- and Cryptophyceae (and Rhodophyceae, which are not members of freshwater phytoplankton); however, some of these algal classes do not produce PE, but, for example, phycocyanine and allophycocyanine instead. This may be a reason, why only a part of the Cyanophyceae individuals were classified into the PE-containing class. Unfortunately, the knowledge about PE and phycocyanine in different Cyanophyceae species is poor. Pigment composition of Dinophyceae varies depending on the species (MacIsaac and Stockner, 1993). The autofluorescence of some Dinophyceae cells interfered with the PE fluorescence in our study.

Some individuals in the samples with low or absent Chl *a* autofluorescence, belonging to species with normally sufficient autofluorescence, probably have low physiological states or were dying. Problems of ambiguous Chl *a* fluorescence information of some algae are not limited to the presented method, but also apply to other methods relying on fluorescence. False positive results due to unspecific, non-Chl *a* or -PE fluorescence were mostly excluded by the use of the ratios.

The interest of this study was strictly focused on the properties of fluorescence. The combination of bright-field (phase contrast) and fluorescence pictures supplies much broader information (e.g. morphometric, densitometric, colourimetric), which further improves separation (Rodenacker *et al.*, 2006). Generally, fluorescence thresholds producing sometimes overestimation of the true algae number are preferred, as false-positive individuals can be eliminated by additional, non-fluorometric classification features.

The second potential source of error involves the equipment used. Depending on the fixation methods, a part of the cells is destroyed. An optimal method for all species does not exist. Debris of ruptured cells may still show Chl *a* or PE autofluorescence.

The excitation light intensity declines during the life span of mercury lamps. The samples used for testing the classification system were taken from a long-term

study on phytoplankton structure development (Rodenacker *et al.*, 2006). During this study, fluorescence images were acquired with different life stages of the mercury lamp, including lamp exchange. The results indicate that the use of FLU1/FLU2 and FLU3/FLU1 ratios rather than FLU1 or FLU3 largely reduce these influences. Nevertheless, an implementation of a fluorescence lamp correction by reference with fluorescent plastic slides (e.g. Chroma Corporation, Brattleborough, VT) could further improve fluorescence quantification.

The fluorescence filter systems were selected to supply information about Chl *a* and PE with respect to their spectra. Other authors recommend different filter combinations (MacIsaac and Stockner, 1993); however, the results in this study were satisfying. Besides these aspects, reasonable classification thresholds depend to some degree on the optical properties of the image acquisition system. Thus, the thresholds in this study can only be regarded as an example, showing that the method is valid. Optimally, threshold setting is adapted to the conditions of each study and interactively controlled. As our underlying image acquisition and analysis system allows this by a direct access to the features of each object (e.g. FLU1, FLU2, FLU3), this can easily be done (Rodenacker *et al.*, 2006).

CONCLUSION

Integrating information from bright-field as well as fluorescence images acquired at the same locations (Walker and Kumagai, 2000; Rodenacker *et al.*, 2006) enables optimized information to be obtained to identify a high number of algal species, partly occurring in low numbers, in a complex matrix containing algae and non-algal species and detritus. This especially applies for samples containing a large proportion of non-algal detritus, which often causes problems in image analysis. This study showed that the use of fluorescence microscopy for discrimination between algal and non-algal objects and PE and non-PE-containing algae supplies stable and reliable results. Thresholds based on fluorescence intensity ratios rather than on fluorescence intensity itself and on mean fluorescence intensities as well as on fluorescence maxima within the cell were necessary for optimal differentiation properties. A critical point is to take care of the fluorescence fading, e.g. by short and adequate sample storage and a suitable image acquisition procedure.

The method presented is based on a well-established strategy, the evaluation of phytoplankton by microscopy. As basic modules (microscope, computer) are mostly present in laboratories, the cost for the device is comparatively limited.

The possible taxonomic resolution attainable by most of the other automated phytoplankton investigation methods is lower than that of image analysis in microscopic images (Poryvkina *et al.*, 2001; Bodemer, 2004; Gregor and Marsalek, 2004). This is enabled by integrated analysis of different features, such as size, morphology, volume and physiological aspects (e.g. by investigation of Chl *a* via autofluorescence). Generally, the higher the demands on (taxonomic) resolution are the more advantages automated image analysis systems offer. Usually, only morphometric and densitometric features have been integrated into image analysis. Additionally, we recommend fluorescence features.

Such features can be used as an integrated part of classifiers for studies on higher taxonomic resolution levels, e.g. as a primary step for identification of phytoplankton from detritus and/or a separation of PE- from non-PE-containing algae before subsequent "species" level identification. Especially problems with detritus have up to now often limited the use of image analysis in algal monitoring. Other potential applications are coarse taxonomic investigations, e.g. by counting total number of algae identified on the basis of fluorescence features alone. Adding fluorescence images into the acquisition procedure does not lengthen the time needed in PLASA relevantly, as the autofocus process, which is most time consuming, is not affected.

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