Geophysica (2000), 36(1–2), 161–168

The Influence of Different Solvents and Extraction Regimes on the Recovery of Chlorophyll *a* from Freshwater Phytoplankton

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(Received: January 2000; Accepted: June 2000)

Abstract

The extraction of chlorophyll a *(Chl*a*) with 90% acetone and 96% ethanol was compared in different Estonian stratified lakes and in polymictic Lake Võrtsjärv. The effect of the extraction regime on the recovery of Chl*a *was tested. On the basis of our results it seems important to use several solvents in parallel when investigating lake with unknown phytoplankton species composition. In lakes of frequent monitoring, the most effective solvent should be estimated in a special study. It is shown that ethanol is more effective solvent than acetone when cyanophytes or diatoms are dominant group of phytoplankton. In case of cyanophytes the extent of difference between these two solvents is higher than with diatoms. The best Chl*a *recoveries were achieved when samples were dried only slightly after filtration (some minutes on filter paper in dark, at room temperature) and then soaked into the solvent straight after that. In case of ethanol extraction, both the 5-min hot (75°C) treatment or 4-h extraction at room temperature can be applied equally. Ethanol extracts can be stored in deep freezer (-20°C) for several weeks before analysis. In case of acetone the best recoveries of Chl*a *were obtained when the short time (4-h) extraction at room temperature was carried out. Drying of filters reduced or increased Chl*a *recoveries in acetone when phytoplankton was dominated by diatoms or cyanophytes, respectively. In most cases there was no remarkable difference between extraction effeciency of ethanol and acetone.*

Key words: Chlorophyll extraction efficiency, ethanol, acetone, extraction regimes, lake phytoplankton

1. Introduction

The extraction procedure is one of the most critical steps in quantitative analysis of phytoplankton pigments. The most commonly used solvents, as acetone, ethanol and methanol, have each their benefits and disadvantages. Acetone is widely recommended to extract chlorophylls for spectrophotometry from marine phytoplankton; e.g. *SCOR-UNESCO* (1966), *Strickland and Parsons* (1972). It has been well known that very low acetone extraction efficiency is obtained when common algae belonging to the Chlorophyceae or Cyanophyceae (*Holm-Hansen and Riemann*, 1978; *Rai*, 1973) and that 90% acetone is unable to inhibit chlorophyllase activity (*Barrett and Jeffrey*, 1971). From

safety perspective the biggest problem with acetone is its high flammability (*Wright et al.,* 1997). Methanol has been considered to be better extractant than acetone for many green algae (*Rai*, 1973) and freshwater cyanobacteria (*Holm-Hansen and Riemann*, 1978; *Jones*, 1977). Contrarily, *Bowles et al.*, (1985) found that methanol resulted in lower recovery of Chl*a* from some freshwater cyanobacteria and *Pechar* (1987) found no differences in these two solvents. The most serious problem of using methanol is its extreme sensitivity to the acidification procedure and its high toxicity (*Arvola,* 1981). Ethanol is the safest extraction solvent and widely used for freshwater plankton. The extraction efficiency of ethanol is reported to be similar with acetone and methanol (*Jespersen and Christoffersen*, 1987; *Webb et al.*, 1992). *Arvola* (1981) has found methanol a slightly more effective extraction solvent than ethanol, but ethanol seems to be a better extraction solvent when the degradation products of chlorophyll must be measured. The large variety of extraction regimes from simple soaking of filter into the solvent to complex procedure of freezing, soaking, grinding and sonication has been recommended and tested by many authors (reviewed by *Marker et al*. 1980; *Wright et al.*, 1997).

The aim of present paper was to compare the recovery of chlorophyll *a* extracted with 90% acetone and 96% ethanol in different Estonian lakes, and to test the effect of the extraction regime on the extraction efficiency in Lake Võrtsjärv.

2. Materials and methods

Samples were collected in 8 Estonian stratified lakes in late July and early August, 1998, fortnightly in large, shallow, and eutrophic Lake Võrtsjärv during 1996 for seasonal analysis, and in L. Võrtsjärv in July 24, 1998, and in May 31, 1999 for special methodological tests. Stratified lakes were sampled from 8 depth horizons, depth integrated (samples taken from 0-3m with 0.5m depth interval and mixed) water was sampled from L. Võrtsjärv.

Samples were filtered on Whatman GF/F filters. In samples from stratified lakes and in seasonal samples from L. Võrtsjärv pigments were extracted in parallel by 90% acetone and 96% ethanol, the soaking of filters in solvent either for 4 hours at room temperature or for 24 hours at +4^{o}C was applied. In methodological experiments, 5 parallel filters were used for each solvent (E – 96% ethanol; A – 90% acetone) and extraction regime:

(A/E)W4 – slightly dried (some minutes on filter paper in dark, at room temperature) moist filter soaked straight to solvent, extracted 4h at room temperature;

(A/E)D4 – completely dry (dried for 6 hours on filter paper in dark, at room temperature) filter soaked straight to solvent, extracted 4h at room temperature;

(A/E)WF – slightly dried moist filter soaked straight to solvent, stored for 1–2 weeks at -20^{o}C;

(A/E)DF – completely dry filter soaked straight to solvent, stored for 1–2 weeks at -20°C;

DF(A/E) - completely dry filter stored for 1-2 weeks at -20°C, then soaked to solvent, extracted for 4h at room temperature;

EH - slightly dried moist filter soaked straight to solvent, hot (75°C) ethanol extraction (5 min) applied.

Extract was centrifuged for 10 min at 3000 rpm, and the Chl*a* concentration was analysed with scanning UV-VIS spectrophotometer Cecil-3000 (Great Britain) according to *BMB* (1979). The Equation (1) of *Jeffrey and Humphrey* (1975) was applied if 90% acetone was used:

$$\text{Chl}a\ (\text{mg/m}^3)=(11.85*E_{max}-1.54*E_{645}-0.08*E_{630})*v * V^{-1} * l^{-1} \quad (1)$$

In case of ethanol the Equation (2) was applied (*Arvola*, 1981):

$$\text{Chl}a\ (\text{mg/m}^3) = 10^3 * E_{max} * v * 83^{-1} * V^{-1} * l^{-1} \quad (2)$$

E – extinction at wavelength indicated by subscript, after subtraction of the extinction at 750nm

E_{max} – maximum extinction at the region of 662–666 nm

v – volume of acetone or ethanol (ml)

l – cell (cuvette) length (cm)

V – volume of filtered water (l).

In the methodical experiment in L. Võrtsjärv the recovery was calculated as each measured concentration divided by maximum Chl*a* concentration achieved at this sampling occasion.

3. Results and discussion

In Lake Vellavere Külajärv as well as in the upper layers of most other lakes, E and A gave quite similar Chl*a* concentrations. In the upper layer of L. Tsolgo Mustjärv, and in L. Pindi Kärnjärv E resulted in much higher recovery than A while in L. Verevi, Nohipalu Valgjärv and Rõuge Kaussjärv the opposite tendency occurred (Fig. 1). According to the unpublished data of Reet Laugaste, cyanophytes were dominating in Lakes Vellavere Külajärv, Tsolgo Mustjärv, Pindi Kärnjärv, Peta, Nohipalu Valgjärv and Verevi while they were completely lacking in L. Rõuge Kaussjärv, the phytoplankton of which was dominated by chlorophytes, chrysophytes and cryptophytes. In L. Kooraste Linajärv chlorophytes were dominating.

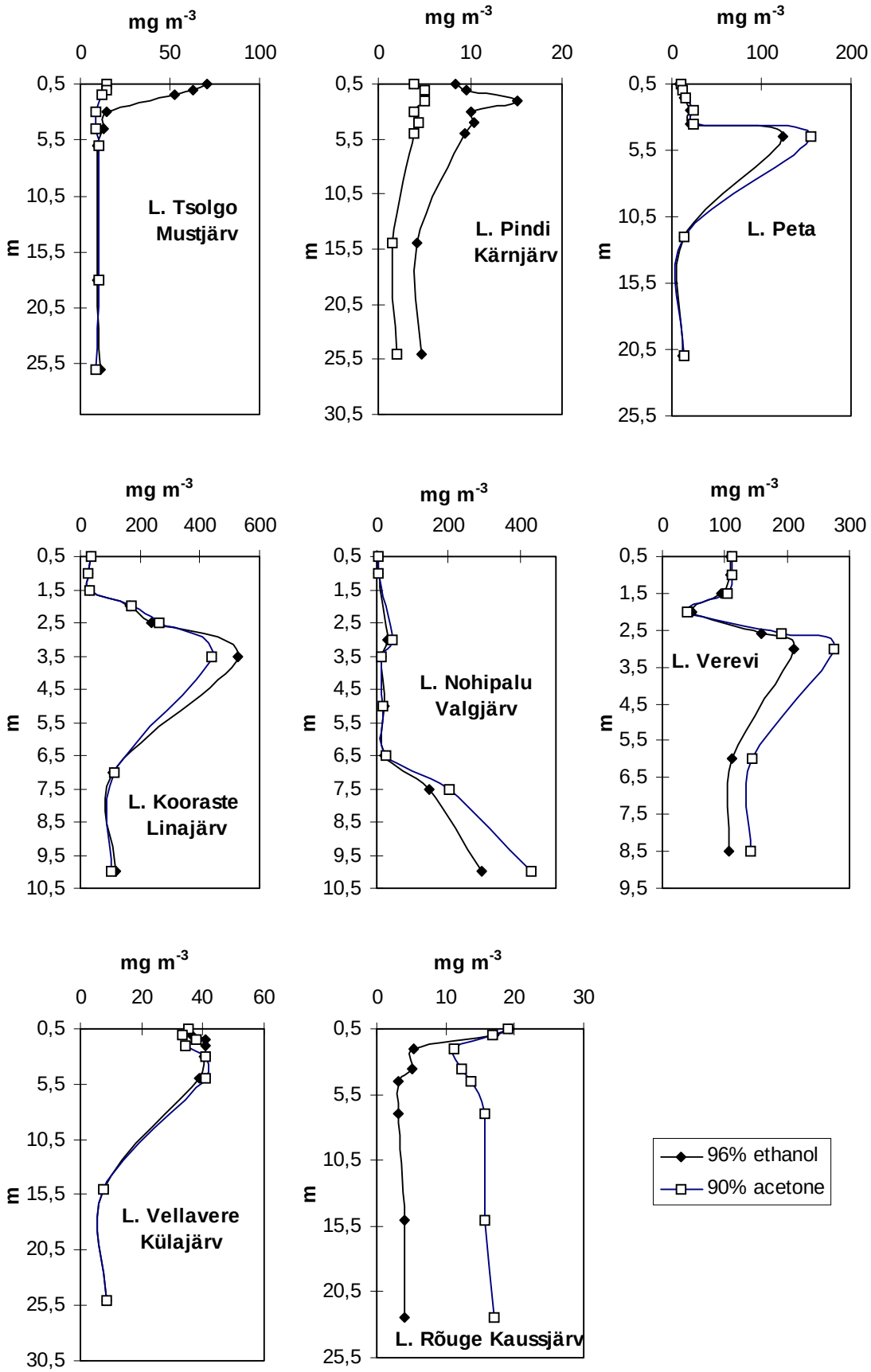

Fig. 1. Chlorophyll *a* concentration achieved using different solvents in Estonian stratified lakes in July and early August, 1998.

In Lake Võrtsjärv cyanophyte *Limnothrix planctonica* was dominating in July, 1998, and diatoms in May, 1999. The experiments in July, 1998 and May, 1999 gave slightly different results (Fig. 2). In July, 1998, acetone extraction resulted much lower recovery than ethanol, especially when slightly dried filters were soaked and stored for two weeks at -20°C. The highest recovery of Chl*a* was achieved when slightly dried filters were soaked straight to ethanol and extraction lasted for 4 h at room temperature or two weeks at -20°C. Drying of filters resulted lower recoveries in case of ethanol, contrarily to acetone, where drying improved Chl*a* recoveries. In May, 1999 the evaluation of different solvents and extraction regimes for L. Võrtsjärv revealed that the highest recovery of Chl*a* was achieved when slightly dried moist filters were soaked straight to solvent. It made no difference weather the extraction lasted for 4h at room temperature or even for several weeks in deep freezer (-20°C). The hot ethanol extraction resulted in slightly (not statistically significant) lower recovery. Drying of filters at room temperature prior to the soaking gave significantly lower recovery in case of E while the difference was not as remarkable when A was used. Acetone gave the highest recovery when slightly dried moist filter was soaked straight to solvent and extracted 4h at room temperature, drying of the filters prior to extraction and keeping of the extract in deep freezer (-20°C) resulted both in slightly lower recovery (Fig. 2).

The parallel extraction of pigments in seasonal samples of L. Võrtsjärv showed that Chl*a* concentration achieved by acetone extraction was 92 ± 9 % (±SD) of that gained by ethanol extraction (Fig. 3).

Our results have shown that the using of only one Chl*a* extraction solvent could cause highly erroneous Chl*a* recoveries because of different extraction efficiencies of different solvents for the extraction of pigments for the different phytoplankton communities of various lakes. It has been also the conclusion of earlier investigators that algal pigments are extracted differentially by various solvents, and there is no single combination of solvent and extraction time best for all species (*Bowles et al.*, 1985). It seems most sensible to use at least two solvents in parallel when investigating lake with unknown phytoplankton species composition and to operate with the maximum concentration obtained. In case of well investigated monitoring lake (as L. Võrtsjärv), the most efficient solvent should be estimated in a special study. On the basis of our study, we were convinced that the use of only slightly dried moist filters which were kept some minutes on filter paper in dark at room temperature and then soaked straight to solvent gave the best Chl*a* recoveries. In case of ethanol extraction the 5-min hot (75°C) treatment or 4h extraction at room temperature can be applied. The recovery of Chl*a* in ethanol extracts was not affected by storage in deep freezer for several weeks before analysis. In case of acetone the short time (4h) extraction at room temperature gave the best results and drying reduced the recoveries of Chl*a* when diatoms were dominating. Contrarily, drying gave higher recoveries when cyanophytes were dominating, probably because of special aid to cell disruption which permittted slightly more effective extraction in acetone.

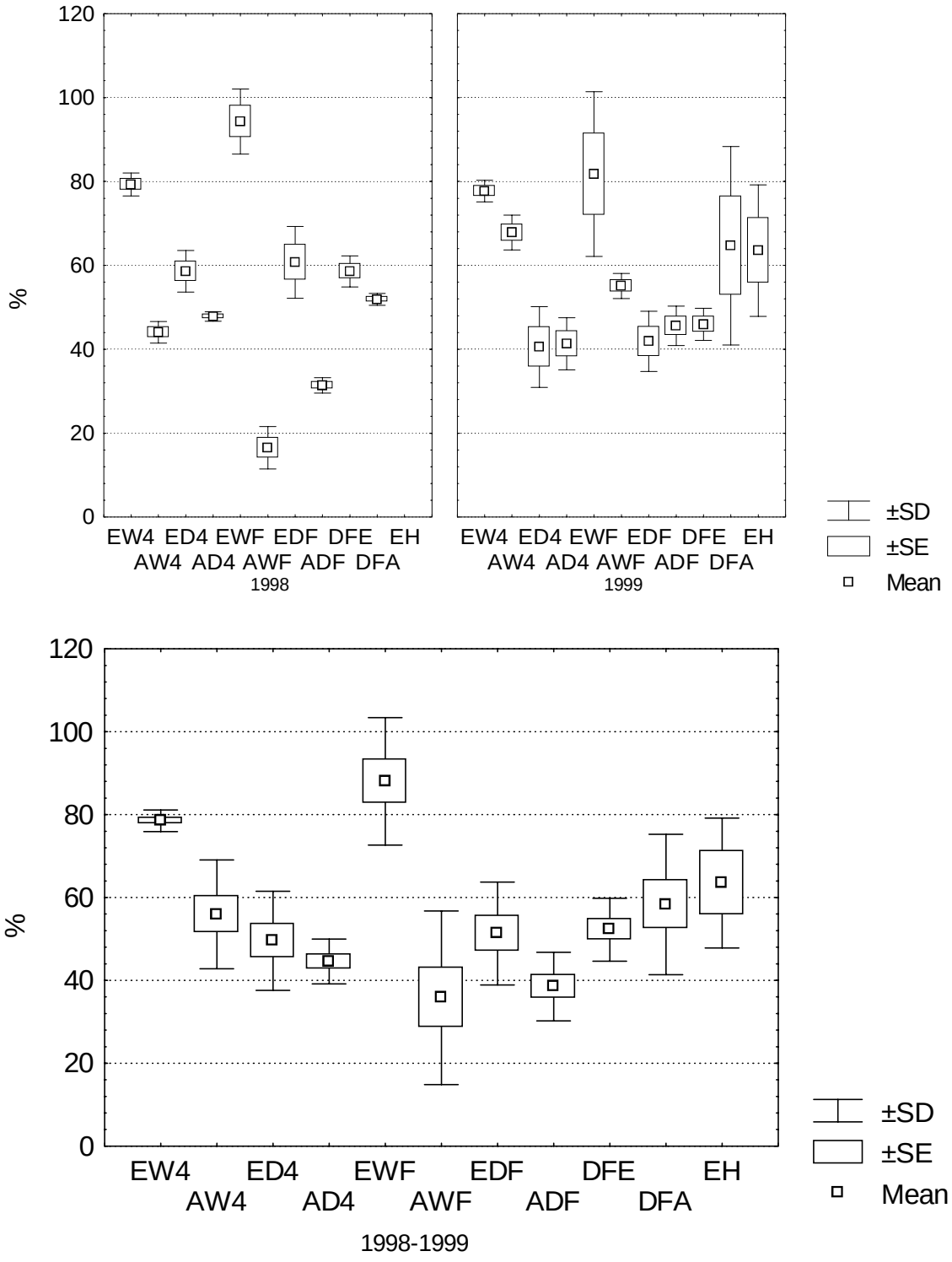

Fig. 2. The recovery of chlorophyll *a* with different solvents and extraction regimes in L. Võrtsjärv in July 24, 1998, and May 31, 1999, and average for both years: (A/E)W4 - slightly dried moist filter straight to solvent, extracted 4h at room temperature; (A/E)D4 - dry filter straight to solvent, extracted 4h at room temperature; (A/E)WF - slightly dried moist filter straight to solvent, 1-2weeks at -20^{o}C; (A/E)DF - dry filter straight to solvent, 1-2weeks at -20^{o}C, DF(A/E) - dry filter 1-2 weeks at -20^{o}C, then to solvent, extracted 4h at room temperature; EH - slightly dried moist filter straight to solvent, hot ethanol extraction (5 min).

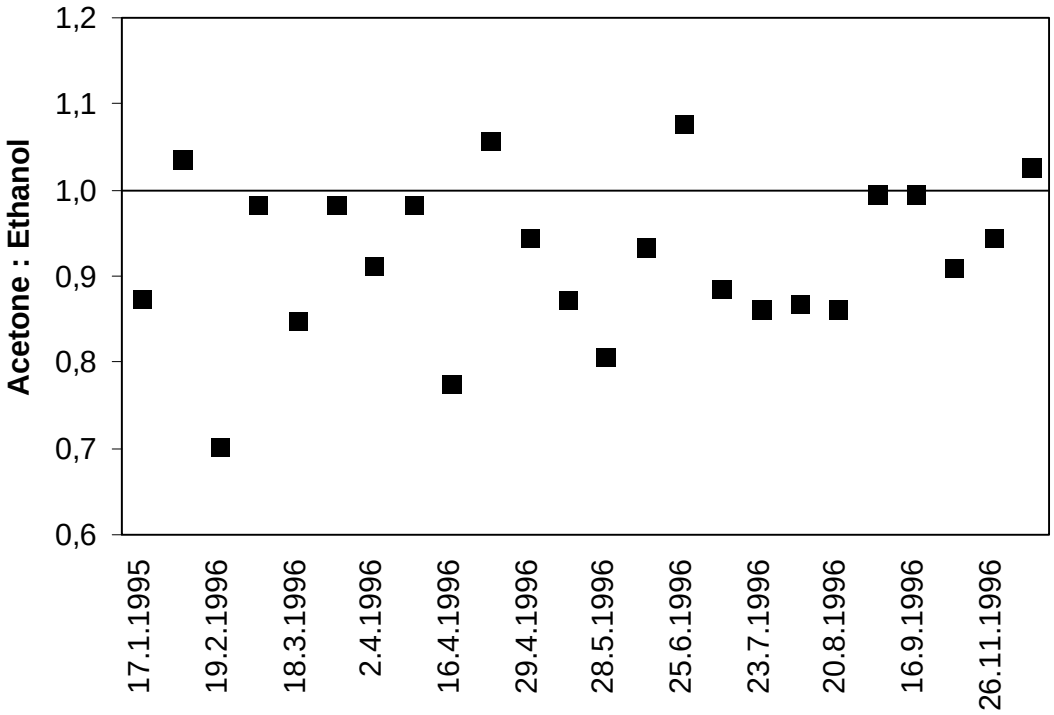

Fig. 3. The ratio of Chl*a* concentration achieved by acetone extraction to that gained at parallel ethanol extraction in L. Võrtsjärv in 1996.

In the upper layers of most of the investigated lakes dominated by cyanophytes or chlorophytes, there were no significant differences between extraction efficiences of ethanol and acetone, except in L. Rõuge Kaussjärv, which was dominated by chlorophytes, cryptophytes and crysophytes, and where acetone gave higher Chl*a* recoveries than ethanol. In two different lakes in which the same genus of phytoplankton was dominating, the extraction efficiences were quite different (e.g. L. Pindi Kärnjärv and L. Peta, both of which were dominated by *Cyanodictyon* sp.).

In the lower layers of L. Verevi where acetone gave higher recovery of Chl*a* than ethanol, photosynthetic sulfur bacteria (as *Thiopedia rosea*, recorded in previous unpublished studies) were assumingly dominated. Similar tendency could be seen in the lower layers of L. Nohipalu Valgjärv and in one strata in L. Peta. The presence of photosynthetic bacteria was, however, not investigated in these lakes.

Acknowledgements

The investigation was supported by Estonian Science Foundation grants No 2017, 4080 and 3579.

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