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Fixation, Counting, and Manipulation of Heterotrophic Nanoflagellates

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Quantitative effects of several fixatives on heterotrophic nanoflagellates (HNAN) and phototrophic nanoflagellates (PNAN) were investigated by hemacytometer and epifluorescence counting techniques. Counts of *Monas* sp. cultures before and after fixation with unbuffered 0.3% glutaraldehyde and 5% formaldehyde showed no loss of cells during fixation, and cell concentrations remained constant for several weeks after fixation. Buffering of fixatives with borax caused severe losses, up to 100% within 2 h. Field samples from Lake Vechten showed no decline of HNAN and total nanoflagellate concentrations for at least 1 week after fixation with 5% formaldehyde and with 1% glutaraldehyde. With 1% glutaraldehyde, the chlorophyll autofluorescence of PNAN was much brighter than with 5% formaldehyde, although it was lost after a few days and thus limited the storage time of samples. However, when primulin-stained slides were prepared soon after fixation and stored at −30°C, the loss of autofluorescence was prevented and PNAN and HNAN concentrations were stable for at least 16 weeks. Effects of filtration and centrifugation on HNAN were also studied. Filtration vacuum could not exceed 3 kPa since 10 kPa already caused losses of 15 to 20%. Similar losses were caused by centrifugation, even at low speed (500 × *g*).

Heterotrophic nanoflagellates (HNAN; size, 2 to 20 μm) are now being recognized as major consumers of bacterial production and as catalysts for mineralization in aquatic ecosystems (4, 9, 18, 21, 31). Quantitative field studies on HNAN and phototrophic nanoflagellates (PNAN) have been permitted by recently developed epifluorescence microscopic counting techniques (2, 5, 10, 20). Since nanoflagellates are very fragile (20, 23), loss of cells as a result of fixation and manipulation before counting must be prevented. Moreover, chlorophyll autofluorescence must be preserved for distinction between phototrophic and heterotrophic cells.

Although various fixatives are used (11, 26, 29), buffered (2, 20, 22) and unbuffered aldehydes are the most common in quantitative studies. The highest final concentration was used by Fenchel (7), who could keep samples fixed with 5% unbuffered formaldehyde for at least 1 month. However, formaldehyde is reported to cause loss of flagella, distortion of cell shape, and loss of cells in many flagellates (16, 27). Some authors recommend a buffered (8, 26) or unbuffered (28) mixture of 1% glutaraldehyde (killing-fixing agent) and 0.03 to 1% paraformaldehyde (preservative) as the best fixative for fragile flagellates. Haas (10) considered a low concentration to be an advantage and used 0.3% glutaraldehyde.

Despite the variability in fixation methods, hardly any quantitative control experiments have been published. Tsuji and Yanagita (28) used the percentage of unbroken cells after fixation as an index for evaluation of fixatives and found 100% unbroken cells in a mixture of 1% glutaraldehyde and 0.03 to 1% paraformaldehyde. However, the presence of perfectly preserved cells does not prove that no losses occur during fixation. Therefore, counts of living flagellates before fixation should be compared with counts at several times after fixation. We know of only one such study, which was performed with electronic particle counters and showed drastic losses of cells in both autotrophic and heterotrophic marine flagellates with all fixatives used (13).

This paper describes quantitative effects of several fixatives on HNAN and PNAN from laboratory cultures as well as freshwater field samples. In addition, effects of filtration and centrifugation on HNAN are discussed.

MATERIALS AND METHODS

Monas sp. (6 to 10 μm) and *Bodo* sp. (6 to 10 μm) were isolated from Lake Vechten, The Netherlands (25), by successive dilutions from enrichment cultures and cultured in 0.025% Knop solution (15) enriched with 100 mg of yeast extract · liter^{-1} (Oxoid). *Chlorella* sp. (3 μm) was isolated from the same lake, and *Haematococcus pluvialis* (20 μm) was isolated from a gutter at our institute. The last two species were cultured in mineral medium no. 11 as described by Allen (1). Field samples from Lake Vechten were obtained with a 5-liter Friedinger sampler from a depth of 4.8 to 6.0 m.

Fixatives at various final concentrations were prepared from stocks of 35 to 38% formaldehyde stabilized with 8 to 13% methanol, 50% glutaraldehyde (Baker grade) in distilled water, and paraformaldehyde powder. In some experiments, fixatives were buffered at pH 7 with 0.01 M phosphate buffer (Na_2HPO_4 plus KH_2PO_4) or at pH 8 with borax ($Na_2B_4O_7 \cdot 10H_2O$) (24) or hexamine (26).

The staining procedure for nanoflagellates was a modification of the method of Caron (2). The fluorochrome primulin (Janssen Chimica, Belgium) was dissolved in distilled water buffered at pH 4 with 0.1 M Trizma hydrochloride (Sigma Chemical Co., St. Louis, Mo.). Initially, a primulin concentration of 250 mg · liter^{-1} was used, which was lowered to 63 mg · liter^{-1} later (see Results). The solution was prepared fresh and was filtered through a 0.2-μm-pore-size membrane filter (Schleicher & Schüll) before each use. Fixed samples (5 to 10 ml) were filtered onto a 1.0-μm-pore-size Nuclepore polycarbonate filter (diameter, 25 mm), prestained with Dylon no. 8 ebony black dye, at a vacuum not exceeding 3 kPa. A 1.2-μm-pore-size

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TABLE 1. Hemacytometer (volume, 0.8 mm^3) counts of living HNAN from three *Monas* cultures

Sample	No. of *Monas* cells/0.8 mm^3[a] for culture:		
	I	II	III
1	141	165	102
2	138	162	85
3	143	174	107
4	140	171	106
5	128	155	115
6	140	175	87
7	133	166	110
8	151	170	109
9	142	164	87
10	142	164	89

[a] For cultures I through III, there were 1.75×10^8, 2.08×10^8, and 1.25×10^8 cells · $liter^{-1}$, respectively.

Schleicher & Schüll membrane filter was used as a backing under the Nuclepore filter. The filter was then rinsed twice with 2 ml of 0.2-μm-filtered Trizma buffer (pH 4.0) and flooded with 2 ml of primulin solution. The vacuum was removed, and the sample was allowed to stain, initially for 15 min and later for 5 min (see Results). The stain was then filtered through, and the filter was rinsed four times with 2 ml of Trizma buffer. The removed filter was mounted with immersion oil between a glass slide and a cover glass. Primulin staining on 0.2-μm Nuclepore filters sometimes resulted in long filtration times and heavy staining of the whole filter surface, making counts impossible. These problems, probably owing to clogging, did not occur with 1.0-μm filters.

The slides were examined at ×1,000 magnification by epifluorescence microscopy in a dark room with a Zeiss Universal II microscope equipped with a Neofluar 100× objective lens, an HBO 50 mercury lamp, and a filter housing (Zeiss 466249-9904) which allowed the rapid exchange of four filter sets. The following filter sets were used: for primulin, a BP 365 exciter filter, an FT 395 beam splitter, and an LP 397 barrier filter (Zeiss filter set 487701); for chlorophyll *a*, a BP 450-490 exciter filter, an FT 510 beam splitter, and an LP 520 barrier filter (Zeiss filter set 487709); and for phycobilins, a BP 546 exciter filter, an FT 580 beam splitter, and an LP 590 barrier filter (Zeiss filter set 487715) (19, 30). For each water sample, at least 100 fields were counted on each of two filters. Usually, between 250 and 500 cells were counted per filter.

Nanoflagellates were centrifuged with an MSE High Speed 18 centrifuge (Measuring & Scientific Equipment Ltd.) equipped with rotor no. 69181 (8 by 50 ml; angle, 30°) at 5°C, and with a Fixette table centrifuge equipped with a swing-out rotor at room temperature.

RESULTS

Counting of living HNAN. To study quantitative effects of fixation, HNAN concentrations before and after fixation should be compared. Therefore, a technique was needed to count living HNAN. This is possible with an electronic particle counter if the medium in which the cells are suspended has a fairly high conductivity, like seawater (12). For freshwater samples, this implies the addition of an electrolyte to a final concentration of at least 0.6%. In two experiments, we added 0.6% Ringer solution (containing, in grams per liter, NaCl, 5.44; KCl, 0.254; $CaCl_2$, 0.109; $NaHCO_3$, 0.121; and citric acid, 0.082, final concentrations) to cultures of *Monas* sp. and found a loss of ca. 20% within 2 h, which increased up to ca. 70% within 20 h. Therefore, freshwater HNAN did not withstand the addition of low electrolyte concentrations and consequently could not be counted with an electronic particle counter.

However, it appeared to be possible to accurately count living, actively swimming HNAN from cultures with a hemacytometer (Table 1). The 95% confidence limits did not exceed 10% of the mean (means ± 95% confidence limits for three cultures were 140 ± 4.4, 167 ± 4.3, and 100 ± 8.2 cells per 0.8 mm^3) if the cell concentrations were in a suitable range (resulting in 5 to 10 cells per field) and counting was performed fast. Each of 10 replicate samples was counted at ×400 magnification within 5 min. If the samples were allowed to stand in the hemacytometer for longer than 10 min, the counts were drastically reduced because the cells migrated to the edges of the cover glass, probably owing to oxygen depletion.

Fixation of cultures. Hemacytometer counts of living HNAN were not possible with field samples because relatively high cell concentrations are required. Therefore, fixation experiments were started with cultures of *Monas* sp. The live cells were counted by hemacytometer before fixation. After fixation, counts were performed by hemacytometer as well as epifluorescence microscopy. In the first experiment, however, the fluorescence counts appeared to be up to 45% lower than the hemacytometer counts. One cause of the differences was the distribution of HNAN over the effective filter area. Near the edges, the HNAN densities per field were as low as 10% of the densities in the central filter area. These low densities may be explained by penetration of the sample under the edges of the filter tower. When the edges were excluded from the calculation, ca. 30% higher HNAN concentrations were obtained. Counting was therefore not started as soon as HNAN were observed but at a distance of 20 fields from the edges.

Another cause of differences was the rinsing procedure with Trizma buffer during staining. When the rinse fluid was sucked down until the meniscus just reached the filter surface (5), lower HNAN concentrations were obtained than when the meniscus was kept 5 mm above the filter. With a *Monas* culture, the first method yielded $(0.53 \pm 0.12) \times 10^8$ cells · $liter^{-1}$ (mean ± standard deviation, n = 2). The second method yielded $(0.80 \pm 0.08) \times 10^8$ cells · $liter^{-1}$ (n = 5), which agreed well with hemacytometer counts of $(0.90 \pm 0.11) \times 10^8$ cells · $liter^{-1}$ (n = 4). Thus hemacytometer and fluorescence counts yielded identical results if the meniscus was not allowed to reach the filter surface during rinsing and if the edges of the filter area were not taken into account.

In the first fixation experiment, three fixatives were tested: 0.3% glutaraldehyde, a borax-buffered mixture of 1% glutaraldehyde and 1% paraformaldehyde (pH 8), and 5% formaldehyde (final concentrations). Hemacytometer counts showed no loss of cells during 4 weeks after fixation with 0.3% glutaraldehyde and 5% formaldehyde. In the buffered mixture, however, 40% of the cells were lost after 2 weeks. Because of the disagreement between hemacytometer and fluorescence counts in the first experiment, it was repeated, with good agreement between the two counting methods. The results were similar to those of the first experiment (Table 2). With 0.3% glutaraldehyde and 5% formaldehyde, the heterotrophic *Monas* cells were well preserved for several weeks, whereas cells in the borax-buffered mixture showed considerable losses.

For distinguishing between heterotrophic and photo-

TABLE 2. *Monas* concentrations before and after fixation[a]

Fixative[a]	Counting method[b]	*Monas* concn (10^8 cells/liter): Before fixation[c]	After fixation[d]: 2 h	1 day	1 wk	2 wk	4 wk	% Loss after 4 wk
A	F		1.15 ± 0.04	1.15 ± 0.00	1.18 ± 0.06	1.19 ± 0.01	1.17 ± 0.01	6
	H	1.25 ± 0.14	1.16 ± 0.04	1.17 ± 0.02	1.19 ± 0.07	1.15 ± 0.04	1.29 ± 0.06	0
B	F		1.11 ± 0.01	1.04 ± 0.08	0.98 ± 0.02	0.97 ± 0.00	0.84 ± 0.02	33
	H	1.25 ± 0.14	1.11 ± 0.05	1.05 ± 0.01	1.05 ± 0.08	1.04 ± 0.06	0.93 ± 0.05	26
C	F		1.20 ± 0.01	1.17 ± 0.01	1.16 ± 0.01	1.18 ± 0.03	1.00 ± 0.06	20
	H	1.25 ± 0.14	1.23 ± 0.04	1.17 ± 0.09	1.24 ± 0.01	1.18 ± 0.04	1.11 ± 0.02	11

[a] Fixatives: A, 0.3% glutaraldehyde; B, 1% glutaraldehyde plus 1% paraformaldehyde, buffered by borax (pH 8.2); C, 5% formaldehyde.
[b] Abbreviations: F, fluorescence; H, hemacytometer.
[c] Values are given as mean ± standard deviation of 10 determinations.
[d] Values are given as mean ± standard deviation of two determinations.

trophic nanoplankton cells in field samples, a good preservation of chlorophyll autofluorescence is also required. This was studied in a third experiment, with a mixture of heterotrophic *Monas* and phototrophic *Chlorella* cells, both from cultures. Because of the previous results, 0.3% glutaraldehyde and 5% formaldehyde were tested again. In addition, phosphate-buffered 0.3% glutaraldehyde (pH 7) and borax-buffered 5% formaldehyde (pH 8) were used. Hemacytometer as well as fluorescence counts showed no considerable loss of *Monas* cells after 4 weeks with three of the four fixatives (Table 3). With borax-buffered formaldehyde, however, all cells were lost within 2 h. The hemacytometer counts showed no loss of *Chlorella* cells during 4 weeks with all fixatives used. The fluorescence counts of *Chlorella* were lower than the hemacytometer counts, probably owing to dead cells, which lack chlorophyll autofluorescence. The chlorophyll autofluorescence was preserved for a maximum of 1 week with 0.3% glutaraldehyde. The phosphate buffer did not improve the results. The autofluorescence was completely lost after 4 weeks. In 5% formaldehyde, the autofluorescence of *Chlorella* cells was preserved for several weeks, although it was hard to see with borax buffer.

Fixation, staining, and storage of field samples. Cultures showed the best preservation of cells and autofluorescence with 5% formaldehyde. With 0.3% glutaraldehyde, cell outlines tended to fade after a few weeks and chlorophyll autofluorescence was lost more rapidly. To obtain a better preservation, the glutaraldehyde concentration was raised to 1% in experiments with field samples, which were counted by epifluorescence microscopy after fixation with 1% glutaraldehyde and 5% formaldehyde (Table 4). HNAN and PNAN were distinguished by chlorophyll autofluorescence. However, some of the cells showed no bright autofluorescence and were classified as doubtful cases. Their autofluorescence was not improved when the fixatives were buffered at pH 8 with hexamine. HNAN and total nanoflagellate concentrations were constant for 1 week with both

TABLE 3. Concentrations of heterotrophic (*Monas*) and phototrophic (*Chlorella*) nanoplankton cells before and after fixation

Fixative[a]	Species	Counting method[b]	Concn (10^8 cells/liter)[c]: Before fixation	After fixation: 2 h	1 day	1 wk	2 wk	4 wk	% Loss after 4 wk
A	*Monas*	F		1.15 ± 0.08	1.23 ± 0.00	1.22 ± 0.01	1.21 ± 0.08	1.02 ± 0.02	15
		H	1.20 ± 0.26	1.17 ± 0.06	1.20 ± 0.00	1.21 ± 0.07	1.21 ± 0.10	1.19 ± 0.09	1
	Chlorella	F		0.89 ± 0.01	0.82 ± 0.00	0.82 ± 0.10	0.55 ± 0.08	0.00 ± 0.00	100
		H	1.18 ± 0.09	1.08 ± 0.01	1.05 ± 0.03	1.08 ± 0.07	1.06 ± 0.04	1.08 ± 0.07	9
Ab	*Monas*	F		1.21 ± 0.02	1.23 ± 0.03	1.20 ± 0.00	1.23 ± 0.01	1.24 ± 0.04	0
		H	1.20 ± 0.26	1.16 ± 0.03	1.20 ± 0.06	1.24 ± 0.03	1.20 ± 0.02	1.19 ± 0.04	1
	Chlorella	F		0.88 ± 0.01	0.78 ± 0.01	0.71 ± 0.01	0.47 ± 0.11	0.00 ± 0.00	100
		H	1.18 ± 0.09	1.05 ± 0.06	1.10 ± 0.08	1.10 ± 0.03	1.06 ± 0.04	1.08 ± 0.04	9
C	*Monas*	F		1.27 ± 0.03	1.23 ± 0.01	1.19 ± 0.04	1.29 ± 0.02	1.21 ± 0.01	10
		H	1.35 ±0.16	1.38 ± 0.01	1.38 ± 0.04	1.38 ± 0.10	1.36 ± 0.10	1.34 ± 0.01	1
	Chlorella	F		1.18 ± 0.10	1.10 ± 0.01	1.03 ± 0.08	1.12 ± 0.01	0.98 ± 0.00	27
		H	1.35 ± 0.18	1.33 ± 0.03	1.27 ± 0.04	1.24 ± 0.06	1.15 ± 0.03	1.18 ± 0.04	13
Cb	*Monas*	F		0.23	0.06	0.07 ± 0.02	0.06 ± 0.01	0.00	100
		H	1.35 ± 0.16	0.00	0.00	0.00	0.00	0.00	100
	Chlorella	F		1.15	0.69	0.89 ± 0.04	0.90 ± 0.04	0.99 ± 0.10	27
		H	1.35 ± 0.18	1.36 ± 0.02	1.29 ± 0.08	1.33 ± 0.13	1.37 ± 0.00	1.18 ± 0.04	13

[a] Fixatives: A, 0.3% glutaraldehyde; Ab, 0.3% glutaraldehyde plus phosphate buffer (pH 7.0); C, 5% formaldehyde; Cb, 5% formaldehyde plus borax buffer (pH 8.2).
[b] Abbreviations: F, fluorescence; H, hemacytometer.
[c] Mean ± standard deviation of two determinations.

TABLE 4. Fluorescence counts of nanoplankton from Lake Vechten (sampling depth: 4.8 to 6.0 m, 7 May 1985) at several times after fixation

Fixative	Cell type	10^6 Cells/liter at time after fixation[a]:		
		1 h	1 day	1 wk
1% Glutaraldehyde	HNAN	2.89 ± 0.01	2.65 ± 0.05	2.73
	PNAN	3.51 ± 0.05	0.97 ± 0.11	0.09
	?NAN[b]	1.15 ± 0.10	4.43 ± 0.23	5.55
5% Formaldehyde	HNAN	2.41 ± 0.32	2.47 ± 0.17	2.49
	PNAN	0.63 ± 0.35	0.37 ± 0.05	0.00
	?NAN[b]	4.24 ± 0.04	4.93 ± 0.27	5.49

[a] Values are given as mean ± standard deviation of two determinations.
[b] ?NAN, Doubtful cases (autofluorescence not clear).

fixatives. However, chlorophyll autofluorescence was lost rapidly, resulting in low PNAN counts and a high fraction of doubtful cases. The brightest autofluorescence of PNAN was observed with 1% glutaraldehyde, although it was lost after 1 day.

The loss of autofluorescence was studied with a cultured phototrophic flagellate, *H. pluvialis*. This species showed bright autofluorescence in water 5 days after fixation with either 1% glutaraldehyde or 5% formaldehyde. Trizma buffer (pH 4.0) did not affect autofluorescence. However, autofluorescence strongly decreased when primulin was added, dissolved either in Trizma buffer or in distilled water. Since primulin seemed to cause loss of autofluorescence, the staining procedure was modified. Reduction of staining time from 15 to 5 min and of primulin concentration from 250 to 63 mg · liter^{-1} strongly reduced the fraction of cells showing doubtful autofluorescence in field samples (Table 5). The ratio of doubtful cases to PNAN was reduced from 0.57 to 0.08, whereas HNAN and total nanoflagellate concentrations were not affected. Thus bright autofluorescence of PNAN was achieved up to 2 days after fixation with 1% glutaraldehyde. After 6 days, the chlorophyll autofluorescence had been lost, although the autofluorescence of phycobilins was still intact. The fixed samples were stored at 5°C in a refrigerator.

Because the chlorophyll autofluorescence of PNAN was preserved for only a few days, the effect of storage in a freezer was studied. Field samples were fixed with 1% glutaraldehyde immediately after being taken, and the staining procedure was performed within 1 day. On the day of sampling (t = 0), 3 slides were counted and 25 slides were stored in a freezer at −30°C. At regular time intervals, three of the slides were taken out of the freezer and nanoflagellates were counted. HNAN and PNAN counts were constant for at least 16 weeks, and no decrease of autofluorescence was observed (Table 6).

Manipulation of HNAN. Regarding the fragility of HNAN and the required filtration in the staining procedure, the effect of filtration vacuum (i.e., the pressure differential over the filter) on HNAN was investigated. Exponentially growing cultures of *Monas* and *Bodo* spp. were fixed with 0.3% glutaraldehyde and counted 10 times by hemacytometer to obtain concentrations without filtration (vacuum, 0.00 kPa). HNAN in field samples could not be counted by hemacytometer because of their lower concentrations. After fixation with 5% formaldehyde, they were gravity filtered on 1-μm Nuclepore filters without vacuum applied (0.00 kPa) and counted in duplicate by epifluorescence microscopy. Further epifluorescence counts were performed of *Monas* sp., *Bodo* sp., and the field samples after filtration on 0.2-μm Nuclepore filters at 3, 10, and 30 kPa, respectively. At a vacuum exceeding 3 kPa, the cultures as well as the field samples showed considerable losses, between 15 and 36%, whereas no significant losses were observed at 3 kPa (Table 7). Therefore, a vacuum of 3 kPa was used as an upper limit for filtration of HNAN.

In addition, the effects of centrifugation on *Monas* and *Bodo* spp. were studied. Samples (15 ml) from exponentially growing cultures were centrifuged alive for 30 min in an MSE centrifuge at forces ranging from 500 × g (2,000 rpm) to 38,000 × g (18,000 rpm). After centrifugation, pellet and supernatant were mixed and the HNAN were fixed with 5% formaldehyde. Fixed samples were counted by hemacytometer before and after centrifugation. Even low-speed centrifugation (500 × g) caused significant losses (18 to 34%) of HNAN (Table 8). High-speed centrifugation (38,000 × g) destroyed 55% of the *Monas* cells and 98% of the *Bodo* cells.

TABLE 5. Effect of staining time and primulin concentration on nanoplankton counts (Lake Vechten, 21 May 1985)

Time after fixation (days)	Primulin concn (mg/liter)	Staining time (min)	10^6 cells/liter		
			HNAN	PNAN	?NAN[a]
1	250	15	7.35	2.62	1.49
1	250	10	6.53	2.95	0.87
1	250	5	7.11	3.24	0.28
2	250	5	6.70	2.93	0.91
2	125	5	6.83	3.70	0.66
2	63	5	7.00	3.84	0.30

[a] ?NAN, Doubtful cases.

TABLE 6. Counts of HNAN and PNAN (Lake Vechten, 12 November 1985) after up to 16 weeks storage in a freezer at −30°C

Storage time	Counts (10^6 cells/liter) of:	
	HNAN	PNAN
0	2.93 ± 0.24	0.96 ± 0.07
1 day	3.09 ± 0.04	0.98 ± 0.06
5 days	2.96 ± 0.12	1.04 ± 0.02
1 wk	3.00 ± 0.18	1.06 ± 0.06
2 wk	2.97 ± 0.12	1.00 ± 0.08
4 wk	2.93 ± 0.08	1.07 ± 0.07
8 wk	2.89 ± 0.07	0.97 ± 0.05
16 wk	2.89 ± 0.13	1.01 ± 0.06

TABLE 7. Effect of filtration vacuum on counts of *Monas* and *Bodo* cultures and HNAN from Lake Vechten

Filtration vacuum (kPa)	Counts (10^8 cells/liter) (% loss) of[a]:		Counts (10^6 cells/liter) (% loss) of HNAN
	Monas sp.	*Bodo* sp.	
0	2.19 ± 0.26[b] (0)	2.28 ± 0.26[b] (0)	3.06 ± 0.25 (0)
3	2.14 ± 0.10 (2)	2.15 ± 0.14 (6)	2.97 ± 0.16 (3)
10	1.79 ± 0.08 (18)	1.82 ± 0.07 (20)	2.60 ± 0.14 (15)
30	1.41 ± 0.04 (36)	1.64 ± 0.04 (28)	2.39 ± 0.30 (22)

[a] Values are given as mean ± standard deviation of two determinations.
[b] Values are given as mean ± standard deviation of 10 determinations.

For experimental purposes, it is desirable to increase HNAN concentrations and to separate HNAN from their growth medium. Therefore, living HNAN were concentrated by filtration, centrifugation, and settling. A 100-ml volume of a *Monas* culture was filtered down to a volume of 10 ml to obtain a 10-fold-higher cell concentration. However, on 1-μm Nuclepore filters, only a twofold concentration was achieved from $(1.39 \pm 0.05) \times 10^8$ to $(3.16 \pm 0.16) \times 10^8$ cells · liter^{-1} (mean ± 95% confidence limits, as counted by hemacytometer). At a vacuum not exceeding 3 kPa, the filtration took 2 to 3 h. Filtration was considerably faster on a 5-μm Nuclepore filter, but this did not raise the cell concentrations at all. Some of the cells passed the 5-μm filters and were observed in the filtrate. Microscopic observations of both 1- and 5-μm filters showed many HNAN sedimented on the filters. These cells could not be resuspended by gentle syringing. To prevent sedimentation of cells and clogging of the filter during filtration, the fluid above the filters was continuously stirred. Despite stirring, it was impossible to filter 100 ml of a *Bodo* culture because 1-μm as well as 5-μm filters became clogged. We were therefore not able to concentrate HNAN by filtration.

Centrifugation for 30 min at 1,000 × *g* in the swing-out rotor of the Fixette centrifuge resulted in the concentration of a *Monas* culture from $(2.37 \pm 0.36) \times 10^8$ to $(8.08 \pm 0.73) \times 10^8$ cells · liter^{-1} (mean ± 95% confidence limits). Since the pellet and the supernatant contained 96 and 4% of the cells, respectively, a good separation was achieved. However, 50% of the cells were lost during centrifugation. Losses were limited to 20% when a *Bodo* culture was centrifuged for 30 min at 500 × *g* in the angle rotor of the MSE centrifuge. Because the pellet contained only 31% of the cells, no good separation was achieved, although the cell concentration was raised from $(2.31 \pm 0.31) \times 10^8$ to $(9.03 \pm 1.38) \times 10^8$ cells · liter^{-1}. In conclusion, HNAN could be concentrated fourfold by low-speed centrifugation, but a good separation was not obtained without severe losses.

No losses occurred when 300-ml volumes of *Bodo* and *Monas* cultures were incubated in 35-cm high-settling cylinders at room temperature in the dark. After 24 h, both species showed a threefold concentration in the bottom 30 ml, but this bottom volume contained only 29% of the *Monas* cells and 55% of the *Bodo* cells and therefore yielded a poor separation.

TABLE 8. Effect of centrifugation on *Monas* and *Bodo* cultures

Centrifugal force (× *g*)	Counts (10^8 cells/liter) (% loss) of *Monas* sp.[a]	Counts (10^7 cells/liter) (% loss) of *Bodo* sp.[a]
0	5.25 ± 0.13 (0)	7.43 ± 0.86 (0)
500	3.45 ± 0.23 (34)	6.06 ± 0.30 (18)
1,000	3.75 ± 0.19 (29)	4.86 ± 1.05 (35)
5,000	4.10 ± 0.24 (22)	2.31 ± 0.21 (69)
10,000	2.82 ± 0.16 (46)	0.74 ± 0.20 (90)
38,000	2.34 ± 0.11 (55)	0.14 ± 0.07 (98)

[a] Values are given as mean ± 95% confidence limits.

DISCUSSION

Fragility of HNAN. HNAN appeared to be very vulnerable to common manipulations such as centrifugation and filtration. Although, as far as we know, no control experiments have been published, different authors used different upper limits for the filtration vacuum. Sherr and Sherr (20) used a vacuum of not more than 0.7 kPa, well below our upper limit of 3 kPa which yielded no losses of HNAN in our experiments (Table 7). However, higher values (7 kPa [10], 13 kPa [2, 3, 31], and 16 kPa [5]) have also been used. At 10 kPa, losses of 15 to 20% were found with cultures as well as field samples. Considerable losses, of at least 20%, were also caused by centrifugation, even at low speed (500 × *g*) (Table 8). Since the *Bodo* sp. showed substantially higher losses than did the *Monas* sp., centrifugation of field samples may cause shifts in species composition. Determination of species composition in field samples requires concentrated samples of live HNAN which can be observed by light microscopy and fixed by electron microscopy. For this purpose, Fenchel (7) centrifuged 0.5-liter water samples until a ca. 1-ml particle concentrate remained. For comparison, we concentrated 15 ml to 1 ml at 500 to 1,000 × *g* and found losses between 20 and 50%. At 1,000 × *g*, *Monas* sp. showed a loss of 29% in the angle rotor (Table 8), whereas 50% of the cells were lost in the swing-out rotor. The higher loss in the latter rotor can be explained by higher hydrostatic pressures which are generated in a swing-out rotor. Despite the risks of centrifugation, it still seems to be the best method available for concentrating HNAN.

Fixation of HNAN. Sherr and Sherr (20) fixed nanoplankton with 2% borax-buffered formaldehyde. The stored samples did not lose counts after 2 weeks, but the total number of heterotrophs and autotrophs did decline, by 46 and 18%, respectively, after 4 weeks. Borax-buffered fixatives also caused losses in our experiments. Within 2 weeks of fixation by a borax-buffered mixture of 1% glutaraldehyde and 1% paraformaldehyde, up to 40% of the *Monas* cells were lost (Table 2). With 5% borax-buffered formaldehyde, and thus a higher borax concentration, all *Monas* cells were completely destroyed within 2 h of fixation, whereas unbuffered 5% formaldehyde did not cause losses (Table 3). Borax tends to produce an internal swelling of crustacea, which makes them turgid (24) and may therefore also cause collapse of nanoflagellates. Cell recovery and chlorophyll autofluorescence were neither decreased nor improved by buffering with hexamine and phosphate buffer. Therefore, buffers were omitted.

Counts before and after fixation with unbuffered 0.3% glutaraldehyde and 5% formaldehyde showed no losses of

Monas cells during fixation, whereas cell concentrations in the stored samples remained constant for several weeks after fixation (Tables 2 and 3). Field samples showed no decline in HNAN and total nanoflagellate concentrations for at least 1 week after fixation with 1% glutaraldehyde and 5% formaldehyde (Table 4). Losses during fixation could not be checked, because live counts before fixation could not be made with field samples. However, instantaneous losses during fixation are not probable, because cell concentrations of *Oxyrrhis marina* and other flagellates decreased only slightly (up to 11%) during and soon after fixation, whereas after 24 h, up to 75% of the cells had disappeared (13). The decrease continued over time.

Klein Breteler (13) and Sorokin (23) concluded that naked protozooplankton cells are hardly preserved, if at all, in common fixatives. Quantitative controls with particle counters showed drastic losses of both heterotrophic and autotrophic flagellates after fixation with low concentrations of acetic acid, trichloroacetic acid, sublimate, benzoic acid-5-hydroxy-sulfo, and Lugol iodine solution (13). According to Taylor (26), Lugol iodine produces a high residue of unidentifiable nanoplankton cells and cannot compare with 2 to 5% glutaraldehyde in its capacity to fix flagella and cell contents. However, Pomroy (17) obtained comparable counts of marine microprotozoans from samples preserved with Lugol iodine and glutaraldehyde. Cultures of fragile *Pavlova lutheri* and *Prorocentrum triestinum* gave 96% of unbroken cells after fixation with 1% glutaraldehyde, whereas reduction of the glutaraldehyde concentration resulted in a considerable decrease in the percentage of unbroken cells (28). This indicates that fixatives at concentrations that are too low may kill cells without fixing them. In our experiments, 0.3 to 1% glutaraldehyde yielded a good fixation.

According to Porter et al. (18), formaldehyde is highly disruptive to soft flagellates and ciliates. The cell shape may become distorted, and flagella may be thrown off in many flagellates (27). Only very weak formaldehyde solutions, with a final concentration below 3%, should be used (26). The concentration of the stock solution of formaldehyde added should not exceed 4%, because the addition of concentrated stock solution to a sample exposes many cells to highly concentrated preservative before mixing is effective, and these cells will be destroyed or become unrecognizable (26). However, as suggested by Fenchel (7), we added concentrated (38%) formaldehyde to our samples; this resulted in a final concentration of 5%, and we found no loss of HNAN cells or flagella. Only cells with visible flagella were counted as HNAN. HNAN and total nanoflagellates in field samples showed similar concentrations after fixation with 5% formaldehyde and 1% glutaraldehyde (Table 4). Although ruminal protozoa may be less vulnerable than planktonic protozoa, the total counts of ruminal protozoa were not affected when the formaldehyde concentration was raised from 4 to 10 or even 18.5% (6). Therefore, no detrimental effects of formaldehyde were observed in our experiments.

Fixation of PNAN. In field samples, the estimate of HNAN concentrations depends upon an accurate estimation of PNAN concentrations (2, 5). Since PNAN cannot always be recognized by shape, a good preservation of chlorophyll autofluorescence is required. Although HNAN could be preserved for several weeks, the autofluorescence of PNAN was lost more rapidly and thus limited the storage time of samples. In field samples, 5% formaldehyde strongly decreased the autofluorescence of PNAN, whereas 1% glutaraldehyde yielded a much brighter autofluorescence and thus higher PNAN concentrations (Table 4, 1 h after fixation). Therefore, 1% glutaraldehyde was used for fixation of field samples. Unfortunately, the autofluorescence was lost after 2 days of storage at 5°C in a refrigerator. A drop in the fluorescence of chlorophyll pigments at 2 days after fixation for samples preserved with 1% formaldehyde was also noted by Davis and Sieburth (5). They used 1% glutaraldehyde when samples had to be counted more than 2 days after fixation. Algae fixed with 5% glutaraldehyde showed sufficient retention of chlorophyll pigments to allow counting after 9 months of sample storage at 9°C (30). The retention time of chlorophyll autofluorescence after fixation depends upon the type of organism. Cultured *Chlorella* sp. and *H. pluvialis* cells, as well as green algae and cyanobacteria in field samples, showed a much longer retention of chlorophyll autofluorescence than did PNAN in field samples from Lake Vechten. The loss of autofluorescence in PNAN was prevented when primulin-stained slides were prepared within 1 day of fixation and then stored in a freezer at −30°C. PNAN and HNAN concentrations were stable for at least 16 weeks (Table 6), and no decrease in the fluorescence intensity was observed. Haas (10) observed significant fading of fluorescence in proflavine-stained slides after 1 to 2 weeks of storage at −15°C. However, Landry et al. (14) observed an excellent condition of nanoplankton cells after 4 months of storage at −15°C: Quantitative data were provided by Tsuji and Yanagita (28), who noted a loss of only 10% of *Pavlova lutheri* cells with red chlorophyll autofluorescence after 2 months of storage at −20°C. Prolonged storage for nearly 6 months did not cause a further decrease in the fluorescent cell number. Their technique was more complicated than ours and involved mounting of filters with glycerol jelly and storage in a desiccator.

Concluding, HNAN were well preserved with 5% formaldehyde as well as 0.3 to 1% glutaraldehyde. With 1% glutaraldehyde, the chlorophyll autofluorescence of PNAN could be preserved for a few days, whereas 5% formaldehyde strongly decreased the autofluorescence. Prepared primulin-stained slides could be stored at −30°C for at least 16 weeks without loss of counts and autofluorescence.

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