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EFFECT OF DIFFERENT FIXATIVES AND PRESERVATIVES ON PHYTOPLANKTON COUNTS

Guy Hällfors[1], Terttu Melvasalo[2], Åke Niemi[1] & Hilkka Viljamaa[3]

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A study was made of the effect of the fixatives/preservatives, Lugol, Lugol+ acetic acid, Keefe and formalin on counts (Utermöhl) of brackish-water phytoplankton stored for 0, 1, 6 and 12 months. The samples were taken from the coastal waters of the Gulf of Finland during 1) the vernal diatom bloom in May, 2) the low production stage in June and 3) in September. Lugol + acetic acid proved to be the best preservative for the present material.

Index words: Phytoplankton, fixatives, preservatives.

1. INTRODUCTION

The joint monitoring programme for the Baltic Sea, starting in 1979, includes qualitative and quantitative phytoplankton studies (Interim Commission 1979). In programmes of this kind it is evident that the results of different scientists and laboratories should be comparable. However, no agreement has been reached concerning the priority of the different fixatives/preservatives used.

In the laboratories studying Baltic Sea phytoplankton different preservatives are used for fixing and preserving quantitative phytoplankton samples. The most commonly used agents are Lugol's solution, with or without acetic acid, Keefe's solution and formalin.

The effect of the preservatives on algae varies with the group and species (e.g. Lund et al. 1958, Paasche 1960, E. Willén 1974, Steemann Nielsen 1975, Unesco 1978). Formalin may destroy certain fragile nanoplankters (Hällfors and Niemi 1974, Steemann Nielsen 1975, Throndsen 1979), and during long storage may dissolve silicate and destroy diatoms with weakly silicified walls (Niemi 1975). In contrast, Lugol and Lugol + acetic acid (Lugol AA) have proved to be suitable for fragile flagellates (T. Willén 1962) and Keefe for abundant blue-green algae in eutrophicated waters (Melvasalo et al. 1973).

1 University of Helsinki, Tvärminne Zoological Station, SF-10850 Tvärminne
2 National Board of Waters, Finland, Box 250, SF-00101 Helsinki 10
3 Helsinki City Water Laboratory, Kyläsaarenkatu 10, SF-00550 Helsinki 55

Too high pH causes dissolution of silicate during storage and too low pH dissolution of calcified structures. It is important to find the preservative which causes the least changes in heterogeneous phytoplankton material (Throndsen 1978), and the aim of the present study was to find the best preservative for Baltic Sea phytoplankton.

The study was part of the Finnish contribution to a more comprehensive investigation on preservatives carried out by the BMB (Baltic Marine Biologists) WG 9 (BMB/WG 9, 1976).

2. MATERIAL AND METHODS

2.1 Sampling

The samples were collected in 1977 (I) during the diatom maximum (May), (II) during the early summer low production stage (June) and (III) in late summer (September), in the coastal waters of the Gulf of Finland.

I May 9, 1977: Outer archipelago off Helsinki (Katajaluoto), slightly eutrophicated. Ca. 50 l surface water (1 m), taken with a water bottle sampler (28 l), was put in a big pail. The sample was mixed continuously by aeration during subsampling.

II June 13, 1977: Outer archipelago of Tvärminne (Storfjärden). Twenty litres of surface water (1 m) was put in a pail. The water was mixed with a scoop during subsampling.

III September 13, 1977: Outer archipelago of Tvärminne (Storfjärden). As on June 13.

Glass bottles of 200 ml were used for storing 40 subsamples of each sample. The subsamples were fixed immediately after subsampling. In addition, one water sample and one net sample (20–25 μm) from each sampling were examined alive. No additional fixatives/preservatives were added to the samples during storage.

2.2 Phytoplankton material

Sample I represented the vernal diatom maximum, consisting chiefly of cold-water diatoms and dinoflagellates and only a few fragile flagellates.

Samples II and III were characterized by higher diversity and a much larger proportion of fragile flagellates.

2.3 Fixatives/preservatives

The following fixatives/preservatives were used:

1. Lugol's solution (Utermöhl 1958)
15 g potassium iodide (KI) dissolved in 50 ml H_2O
7–10 g iodine (I_2)
add distilled water to a final volume of 500 ml

2. Lugol's solution + acetic acid (T. Willén 1962)
20 g potassium iodide (KI)
200 ml distilled water
10 g iodine (I_2)
20 g acetic acid (CH_3COOH)

3. Keefe's solution (Keefe 1926)
900 ml 50 % ethanol (C_2H_5OH)
50 ml formalin (40 % formaldehyde (HCHO)
25 ml glycerine ($C_3H_5(OH)_3$)
25 ml acetic acid (CH_3COOH)
100 g cupric chloride ($CuCl_2$)
15 g uranic nitrate ($UO_2(NO_3)_2 \cdot 6(H_2O)$)

4. Neutralized formalin (Unesco 1978)
500 ml formalin (40 % formaldehyde HCHO)
100 g hexamethylenetetramine ($C_6H_{12}N_4$)
filtrate after one week, add 500 ml distilled water

The amounts of preservatives used for a 200 ml bottle were: Lugol 0.5 ml, Lugol AA 0.5 ml, Keefe 10 ml, and neutralized formalin 4 ml.

2.4 Counting

The persons taking part in these studies examined the living material together, to ensure that the taxonomical treatment would be uniform. The subsamples were counted within one week after sampling, and after storage of 1, 6 and 12 months.

The subsamples to be counted were brought to room temperature 24 h before sedimentation, and sedimented for 48 h in the dark. The size of the sedimentation chamber used differed according to the amount of algal material in the samples, but was the same for parallel subsamples. Counting was performed with an inverted microscope according to Utermöhl (1958).

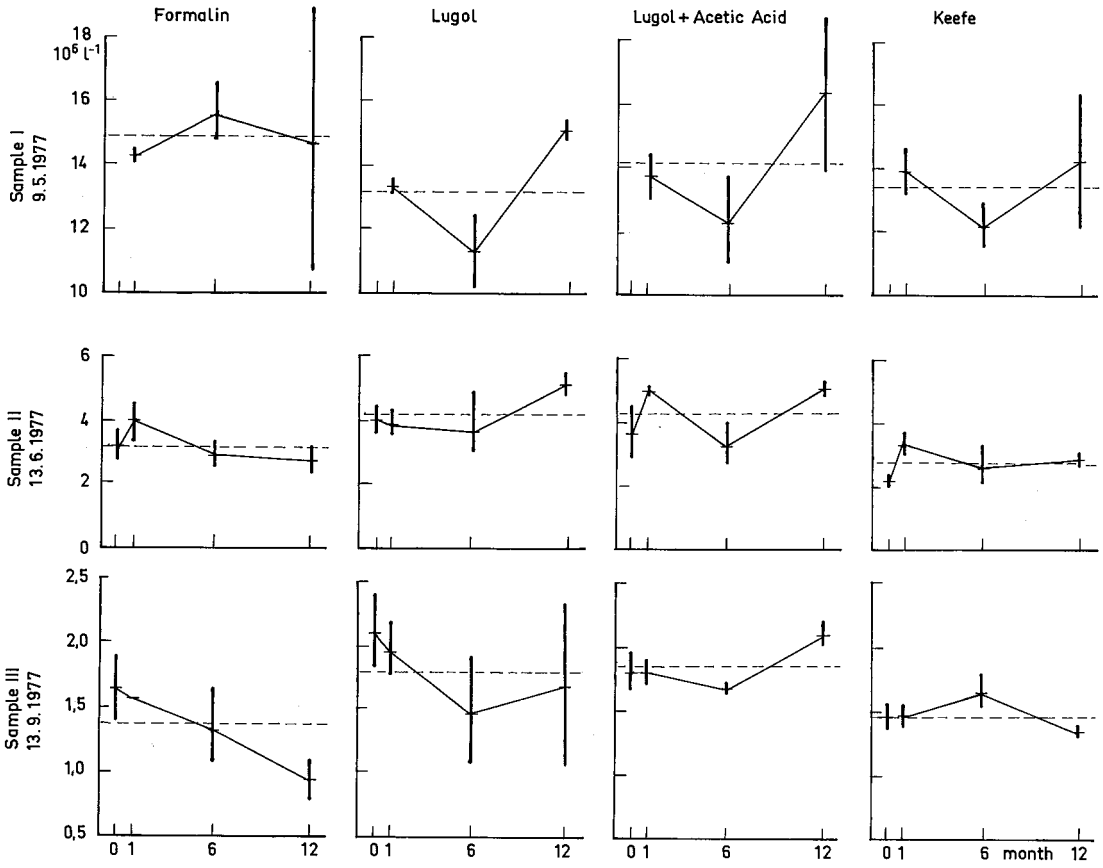

Fig. 1. Relationships between preservation time (month) and the phytoplankton counts (total no. of ind.). The value for 0 month is the mean of four and those of the other months the means of two replicate subsamples (bars = standard deviation).

The sample from Helsinki was counted in the Water Conservation Laboratory of Helsinki City by Maija Huttunen using the random visual field technique and counting all the species with one magnification, 625 x. Sedimentation chambers of 10 ml were used. In every subsample 900–1 000 units were counted. This method is described and the mean volumes of species are given in Melvasalo et al. (1973).

Samples II and III from Tvärminne were counted at the National Board of Waters by Pirkko Kokkonen. Sedimentation chambers of 50 ml were used and the small species were counted on strips using a magnification of 800 x. The large species were counted with a magnification of 200 x on half of the bottom of the chamber. The biomasses were calculated using the mean volumes of species given in the tables of Naulapää (1972) and Melvasalo et al. (1973).

2.5 Statistical treatment

The subsample bottles to be used for the different preservatives, and counts (after storage of 0, 1, 6 and 12 months) were determined at random. The numbers of parallel subsamples are given in Tables 1–3.

The results were processed to obtain the total number of individuals, total biomass and the densities of certain dominant groups or species.

The 0 month subsamples of sample I were

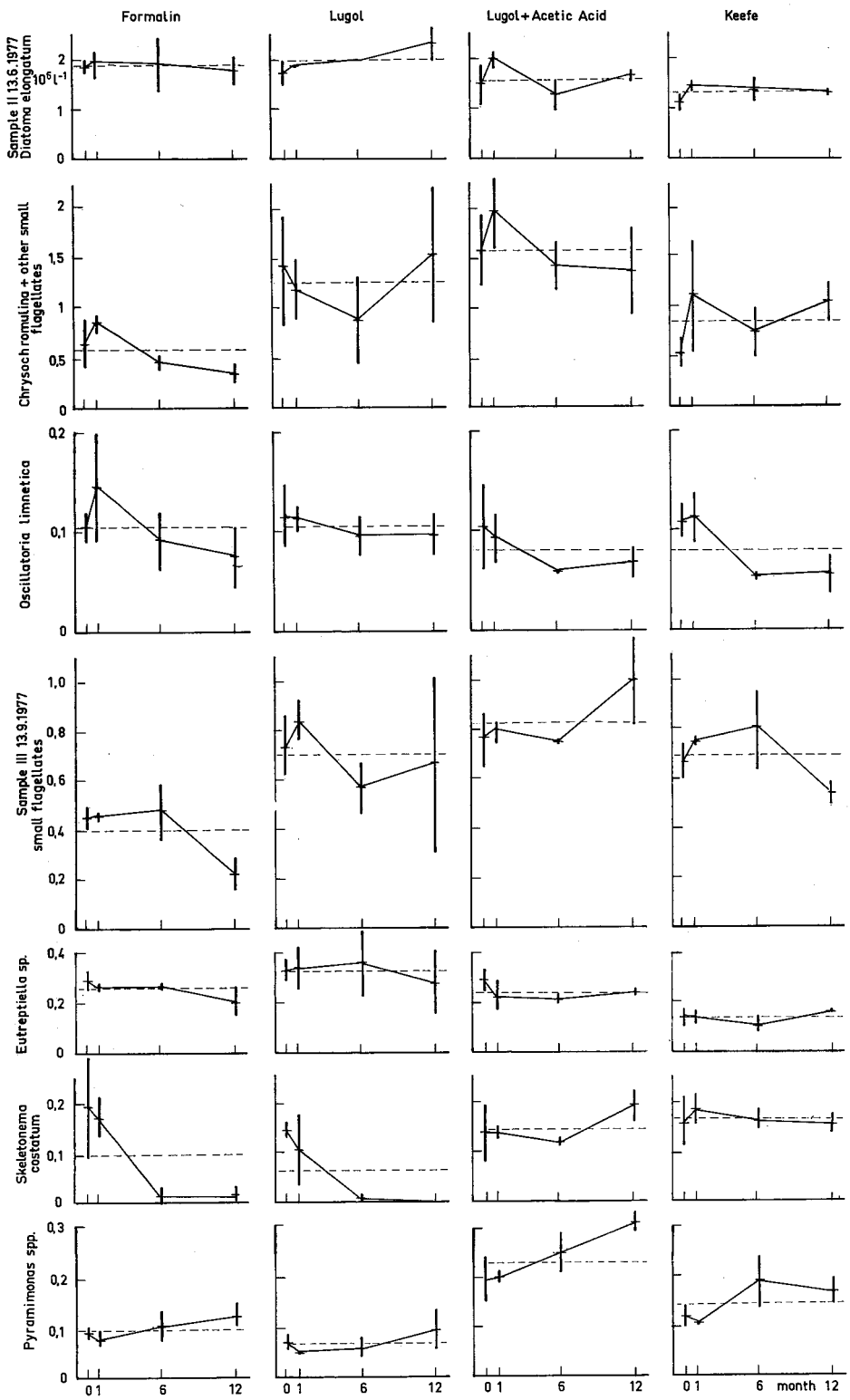

Fig. 2. Relationships between preservation time (month) and the phytoplankton counts (no. of ind.) of some species (bars = standard deviation).

Table 1. Sample I: Katajaluoto 9.5.1977. Total number of individuals, total biomass and densities of some dominant taxa. Means of parallel subsamples, means of preservatives ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV).

Fixative/ preserva-			Tot. no. ind. 10^6/l		%	Tot. biomass mg/l		%	Chaetoceros wighami 10^6/l		%	C. holsaticus 10^6/l		%	Thalassiosira 10^6/l		%	Pyramimonas spp. 10^6/l		%
tive	Month	n	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV
Formalin	1	2	14.27	0.25	1.7	19.36	3.68	19	3.55	0.74	21	2.22	0.20	9.0	2.67	0.43	16	0.29	0.01	3.4
	6	2	15.59	0.90	5.8	19.46	0.55	2.8	4.04	0.25	6.2	3.01	0.40	13	2.69	0.02	7.9	0.36	0.03	8.3
	12	2	14.74	4.10	28	14.16	1.07	7.6	3.38	0.93	27	2.49	0.45	18	3.11	0.14	4.5	0.45	0.04	8.9
	$\bar{x}$ 1–12	3	14.87	0.66		17.66	3.03		3.65	0.34		2.57	0.40		2.82	0.25		0.37	0.08	
Lugol	1	2	13.32	0.23	1.7	17.33	5.84	34	2.51	0.35	14	2.56	0.05	1.9	2.91	0.28	9.7	0.44	0.19	43
	6	2	11.31	1.14	10	13.22	1.19	9.0	1.55	0.18	12	1.87	1.07	57	2.67	0.73	27	0.55	0.06	11
	12	2	15.07	0.32	2.2	16.36	1.04	6.4	1.91	0.80	42	3.63	0.51	14	3.51	0.06	1.6	0.54	0.04	7.4
	$\bar{x}$ 1–12	3	13.23	1.88		15.64	2.15		1.99	0.48		2.69	0.89		3.03	0.43		0.51	0.06	
Lugol AA	1	2	13.68	0.71	5.2	13.47	1.06	7.9	1.90	0.39	21	2.91	0.54	18	2.55	0.32	13	0.42	0.06	14
	6	2	12.33	1.38	11	12.96	3.08	24	1.53	0.39	25	2.37	0.01	0.6	2.74	0.20	7.5	0.48	0.13	27
	12	2	16.31	2.48	15	15.49	0.23	1.5	2.47	0.00	0.0	4.05	1.04	26	2.81	0.33	12	0.76	0.02	2.6
	$\bar{x}$ 1–12	3	14.11	2.02		13.97	1.34		1.96	0.47		3.11	0.86		2.70	0.13		0.55	0.18	
Keefe	1	2	13.86	0.69	5.0	16.38	1.38	8.4	2.27	0.65	29	2.87	0.42	14	2.26	0.04	1.6	0.28	0.11	39
	6	2	12.12	0.74	6.1	13.40	0.24	13.4	1.35	0.30	22	2.27	0.09	4.0	2.53	0.37	15	0.27	0.13	48
	12	2	14.16	2.15	15	14.10	4.56	32	2.37	0.88	37	2.05	0.10	4.8	2.52	0.29	11	0.46	0.01	2.3
	$\bar{x}$ 1–12	3	13.38	1.10		14.63	1.56		2.00	0.56		2.40	0.42		2.44	0.15		0.34	0.10	

Table 2. Sample II: Tvärminne 13.6.1977. Total number of individuals, total biomass and densities of some taxa. Means of parallel subsamples, means of preservatives ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV).

Fixative/ preservative			Tot. no. ind.			Tot. biomass			Diatoma elongatum			Chrysochromulina+ flagellates			Monoraphidium contortum			Oscillatoria sp.		
			10^6/l		%	mg/l		%	10^3/l		%	10^3/l		%	10^3/l		%	10^3/l		%
	Month	n	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV
Formalin	0	4	3.23	0.39	12	1.94	0.09	1.1	1 841	160	9	655	240	37	184	51	28	105	17	16
	1	2	3,89	0.62	16	2.73	0.69	25	1 907	331	17	866	112	13	257	12	5	145	54	37
	6	2	2.94	0.40	14	2.05	0.37	18	1 902	569	30	459	75	16	180	27	15	92	29	31
	12	2	2.73	0.40	15	1.72	0.13	7.8	1 765	332	19	337	112	36	241	0.3	0.0	74	30	41
	$\bar{x}$ 1–12	4	3.20	0.51		2.11	0.43		1 854	66		579	232		216	39		104	30	
Lugol	0	4	4.05	0.38	9.4	2.24	0.26	12	1 688	279	16	1 385	554	40	205	61	30	116	31	27
	1	2	3.91	0.40	10	2.13	0.22	10	1 835	21	1	1 176	295	25	234	21	9	114	13	12
	6	2	3.66	0.61	17	2.14	0.21	11	1 955	21	1	879	440	50	262	11	4	95	21	22
	12	2	5.16	0.33	6.4	2.59	0.19	7.2	2 325	360	16	1 518	688	45	245	11	4	96	17	18
	$\bar{x}$ 1–12	4	4.20	0.66		2.28	0.22		1 951	272		1 240	278		236	24		105	11	
Lugol AA	0	4	3.73	0.77	21	1.64	0.27	16	1 445	415	29	1 573	359	23	180	59	33	104	43	41
	1	2	4.98	0.16	3.2	2.53	0.68	27	1 935	148	8	1 952	351	18	231	34	15	93	24	26
	6	2	3.35	0.71	21	1.66	0.56	34	1 275	332	26	1 434	226	17	200	73	36	62	1	2
	12	2	5.07	0.25	44	2.61	0.51	20	1 640	85	5	1 380	434	32	238	29	12	68	16	24
	$\bar{x}$ 1–12	4	4.29	0.87		2.11	0.53		1 574	283		1 585	258		212	27		82	20	
Keefe	0	4	2.21	0.20	9.1	1.30	0.12	8.9	1 090	151	14	540	142	26	88	29	33	111	15	14
	1	2	3.35	0.34	10	1.63	0.17	10	1 460	45	3	1 114	558	50	188	50	26	113	24	22
	6	2	2.72	0.59	22	1.65	0.31	19	1 376	267	19	754	252	33	147	45	31	52	6	11
	12	2	2.94	0.25	8.5	1.69	0.26	15	1 291	30	2	1 054	191	18	124	19	15	55	18	33
	$\bar{x}$ 1–12	4	2.81	0.47		1.57	0.18		1 304	159		865	268		136	42		83	34	

Table 3. Sample III: Tvärminne 13.9.1977. Total number of individuals, total biomass and densities of some taxa. Means of parallel subsamples, means of preservatives ($\bar{x}$), standard deviation (SD) and coefficient of variation (CV).

Fixative/ preservative	Month	n	Tot. no. ind. 10^3/l		%	Tot. biomass mg/l		%	Flagellates 10^3/l		%	Eutreptiella sp. 10^3/l		%	Pyramimonas spp. 10^3/l		%	Skeletonema costatum 10^3/l		%
			$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV
Formalin	0	4	1 658	274	16	1.73	0.26	15	442	42	9.5	289	41	14	89	7.9	8.9	195	106	55
	1	2	1 565	7	0.0	1.61	0.08	5.0	454	12	2.7	268	15	5.5	80	15	19	176	40	22
	6	2	1 365	304	22	1.46	0.13	8.8	471	115	24	274	12	4.5	104	33	32	16	23	141
	12	2	950	170	18	1.01	0.21	21	222	70	32	205	61	30	123	22	18	16	22	141
	$\bar{x}$ 1–12	4	1 385	314		1.45	0.32		397	117		265	46		99	19		101	98	
Lugol	0	4	2 100	294	14	2.12	0.29	14	732	131	18	332	42	13	72	16	22	143	13	9.4
	1	2	1 950	198	10	2.00	0.33	17	844	86	10	339	86	25	53	2.8	5.3	106	71	68
	6	2	1 490	424	28	2.08	1.00	48	560	106	19	361	137	38	62	18	29	6.5	9.2	141
	12	2	1 680	651	39	1.59	0.69	43	663	365	55	280	128	46	96	40	41	0.0	0.0	
	$\bar{x}$ 1–12	4	1 810	272		1.95	0.24		700	119		328	34		71	19		64	72	
Lugol AA	0	4	1 790	171	9.6	1.86	0.18	9.7	761	105	14	288	50	17	194	45	23	138	54	39
	1	2	1 790	110	6.3	1.64	0.25	15	786	42	5.3	228	57	25	198	7.8	3.9	138	12	8.7
	6	2	1 675	35	2.1	1.52	0.08	5.0	750	11	1.4	220	15	6.8	251	37	15	120	5.7	4.7
	12	2	2 130	106	5.0	1.81	0.02	0.8	994	178	18	242	13	5.6	319	21	6.6	187	31	17
	$\bar{x}$ 1–12	4	1 840	196		1.71	0.16		823	115		245	30		241	58		146	29	
Keefe	0	4	1 437	107	7.5	1.14	0.09	7.9	671	69	10	143	45	32	118	19	16	158	53	34
	1	2	1 455	78	5.3	1.24	0.18	14	753	24	3.2	140	34	25	112	2.2	2.0	183	33	18
	6	2	1 630	127	7.8	1.17	0.01	0.4	802	163	20	113	28	25	188	47	25	164	19	11
	12	2	1 350	42	3.1	1.23	0.03	2.4	541	54	10	165	4.4	2.7	170	26	15	153	19	12
	$\bar{x}$ 1–12	4	1 468	117		1.19	0.05		692	114	16	141	35		147	38		165	13	

lost. The following values were calculated for each sample:

- Arithmetical mean ($\bar{x}$)
- Standard deviation (SD)
- Coefficient of variation (CV) in per cent

Analysis of variance with crossed classification (ln transformation) was applied to ascertain the variations between the results for different preservatives and storage times. The Student-Newman-Keuls test (SNK test) was used to test the means after the analysis of variance (cf. Snedecor and Cochran 1965).

3. RESULTS AND DISCUSSION

I May 9, 1977

Total number of individuals and total biomass: Analysis of variance and the SNK test did not reveal any significant difference between the preservatives.

In the Lugol-fixed sample, the number of individuals was significantly smaller after 6 months than after 1 and 12 months (SNK test).

Chaetoceros wighami: The numbers of individuals were significantly greater in formalin than in the three other preservatives, between which no significant differences were found (SNK test). This might be due to the cells settling closer to the bottom of the counting cell with formalin, and thus being more easily observed.

Pyramimonas spp.: The numbers of individuals were significantly smaller in Keefe and formalin than in Lugol AA and Lugol (SNK test).

The total number of individuals and the densities of the major species showed no significant differences in variance, except that between formalin and Lugol AA for *Pyramimonas* spp. (F-test). No significant differences were found in *Achnanthes taeniata, Chaetoceros holsaticus, Thalassiosira* sp., *Gonyaulax catenata* and small flagellates.

II June 13, 1977

Total number of individuals: Preservation in Keefe gave significantly smaller numbers than the three other preservatives. Formalin gave a smaller number of individuals than Lugol and Lugol AA (SNK test).

Total biomass: Keefe gave smaller biomass values than the three other preservatives (SNK test).

In Lugol-fixed and Lugol AA-fixed samples, the number of individuals was significantly greater after 12 months than after 6 months (SNK test).

In Keefe-fixed samples, the number of individuals was significantly greater after 1 month than after 0 month (SNK test).

The total number of individuals and the densities of the major species showed no significant differences in variance (F-test), except that between formalin and Lugol for *Chrysochromulina* spp. and other naked flagellates. The high variance of Lugol was due to one strongly deviating count.

Lugol and Lugol AA fixed *Chrysochromulina* spp. and other naked flagellates better than formalin and Keefe, Lugol AA apparently being the best.

III September 13, 1977

Total number of individuals: Preservation in Lugol and Lugol AA gave significantly higher numbers of individuals than formalin and Keefe (SNK test).

Total biomass: Lugol gave greater biomass values than Keefe and formalin. Keefe gave significantly smaller biomass values than the other three preservatives (SNK test).

Eutreptiella sp.: Keefe gave a smaller number of individuals than the three other preservatives. Lugol gave a greater number of individuals than the rest (SNK test).

Flagellates: Formalin gave a lower number of individuals than the three other preservatives, between which no significant differences were found (SNK test), although the mean for Lugol AA was somewhat greater than for Lugol and Keefe.

Flagellates in formalin: The number of individuals after 12 months was significantly lower than after 0, 1 and 6 months.

Total individuals and the densities of the

major species showed greater differences than in previous samples (analyses of variance). In many cases Keefe showed the smallest variation (total individuals, total biomass, *Eutreptiella* sp. and *Skeletonema costatum).* This is due to the fact that certain fragile nanoplankters, which are barely recognizable in Lugol and Lugol AA and thus subject to large counting errors, become quite unrecognizable in Keefe (and also often in formalin), which reduces variation. This is in agreement with E. Willén's (1974) results for freshwater nanoplankton.

The dissolution of silicate during preservation in formalin and Lugol destroyed the fragile *Skeletonema* cells, thus causing a decrease in counted individuals with time and an increase in variation.

The smallest differences between the preservatives were obtained with the May sample, which was collected during the vernal diatom bloom. The fairly heavily silicified diatoms predominating in this sample were rather indifferent to the various preservatives. In June and September fragile nanoplankters and weakly silicified diatoms were of great importance which emphasized differences in the effects of the preservatives.

No clear trends were evident as regards preservation time. The variation between the small number of parallel samples possibly overshadowed variation with time. Only *Oscillatoria* sp. (sample II, 13.6.1977) with all the preservatives, and *Skeletonema costatum* (sample III, 13.9.1977) with formalin and Lugol, showed a consistent decrease in counts with time. Differences with time and preservatives have earlier been found in some other species (Maija Huttunen, unpublished).

The variation caused by the small material and inherent weaknesses in the counting technique is probably so large that it masks the actual changes with time.

4. CONCLUSIONS

It is evident that the choice of preservative markedly influences the counts and biomasses obtained in quantitative phytoplankton investigations. The present results were based on phytoplankton material from unpolluted coastal waters. Unfortunately the study did not include samples dominated by blue-green algae, typical of the Baltic Sea phytoplankton. On the basis of our results the preservatives were ranked in the following order of suitability 1) Lugol AA, 2) Lugol, 3) Keefe and formalin.

Lugol AA often gave the highest counts, especially for fragile flagellates (e.g. *Chrysochromulina* spp., *Pyramimonas* spp.). For calcified flagellates (coccolithophorids) Lugol AA is not suitable, as it dissolves the coccoliths, making them unrecognizable (Throndsen 1978). However, this is not a great drawback, at least in the northern Baltic Sea, because this group plays a minor role.

Lugol seemed to be as good or almost as good as Lugol AA, except in some cases with fragile flagellates. However, due to its higher pH Lugol dissolves silicate more rapidly so that weakly silicified diatoms are destroyed within some months. Samples preserved with iodine need attention during storage as iodine is oxidized with time (Unesco 1978).

Keefe gave the lowest counts in many cases, due to poor fixation of fragile flagellates. However, Keefe has proved to be suitable for preservation of phytoplankton dominated by blue-green algae in eutrophicated brackish waters (Melvasalo et al. 1973), owing to better sedimentation of blue-greens with gas vacuoles.

Formalin gave uneven results. Some flagellate species were well preserved, some others not at all. Fixation and preservation varied even within the same genus (e.g. *Chrysochromulina),* evidently depending upon the species in question and external conditions (e.g. temperature, salinity; Guy Hällfors unpubl.). The quality of the formalin probably also influences the fixation and preservation (Unesco 1976). Furthermore, according to our experience in formalin blue-green algae with gas vacuoles do not sediment sufficiently well (e.g. Lund et al. 1958, Hobro and Willén 1977). As with Lugol, weakly silicified diatoms tended to dissolve with time.

LOPPUTIIVISTELMÄ

Kasviplanktonin kestävöinti- ja säilöntäaineiden,

Lugol, Lugol + AA, Keefe ja formaliini vaikutuksia laskentatuloksiin tutkittiin 0, 1, 6 ja 12 kuukauden näytteiden säilytyksen jälkeen. Näytteet otettiin Suomenlahden rannikolta keväisen piilevämaksimin aikaan toukokuussa, matalan tuotantojakson aikaan kesäkuussa sekä syyskuussa. Sinilevävaltaisia näytteitä ei aineistoon sisältynyt. Tutkitussa aineistossa antoi parhaat tulokset Lugol + AA.

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