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Distribution of Bacteria in a Biofilter-equipped, Semi-closed Intensive Fish Culture Unit

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Abstract

Microbial studies were done on a semi-closed recycling intensive fish culture system, equipped with an aerobic (biofilter) and an anaerobic (denitrifier) water treatment units. Total number of bacteria (TBN), the frequency of dividing bacteria cells (FDC) and the percentage of bacteria showing respiratory activity (as measured by INT-reaction) were determined at nine sites in the culture system. The bacteria population of both the aerobic and anaerobic treatment units were also compared using scanning electron microscopy.

TBN differed by orders of magnitude between sample sites in spite of intensive hydrolic mixing within the system (e.g. 10^6 bacteria ml^{-1} in water flowing into the fish basin and 10^{10} bacteria ml^{-1} in the sand-water mixture within the anaerobic treatment unit). Dividing bacteria cells (FDC) were difficult to detect anywhere in the system, even where high loads of organic material should provide abundant bacterial nutrients.

Water from the outflow of the aerobic treatment unit showed similar results of TBN (1×10^8 bacteria ml^{-1}) and portions of INT-active bacteria (12 - 13%) as did effluent from the denitrifying unit. INT-active bacteria were most abundant inside the

biofilter (18% of TBN) and in fish basin effluent (19% of TBN). Microbial parameters varied only slightly during a 24 h cycle.

In the two treatment units microbial colonization differed in both composition and quantity. Probable colonies of ammonia-oxydizing bacteria were detected within the biofilter. No such colonies were seen in the anaerobic unit.

Nitrogen compounds (NH_4-N, NO_2-N and NO_3-N) were sampled simultaneously at three sites. Ammonia showed a periodicity corresponding to the expected metabolic activity of the fish, varying from 0.02 - 0.46 mg L^{-1} in the morning to 0.54 - 2.45 mg L^{-1} in the evening. During the 24 h cycle transformation of NH_4-N through NO_2-N to NO_3-N was inefficient (1.0 - 3.5 mg L^{-1} NO_2-N and 5.0 - 23.6 mg L^{-1} NO_3-N). For their efficient removal, both the aerobic, but especially the anaerobic, treatment units should be optimized. Neither the additions of fish feed or fluctuations in ammonia levels had a noticeable effect on the total number of bacteria or the proportion of dividing or respiring bacteria cells. However, some specialized groups of microorganisms, may have been affected.

KEYWORDS: bacteria, distribution, biofilter

Introduction

Investigations of bacteria in aquaculture systems have largely been neglected in the past (cf. Rosenthal et al., 1988), with the exception of fish pathology for which extensive data exist. This concerns strains and bacteria groups and their possible effects on cultured species and the receiving water body (Austin and Austin, 1987). In aquaculture, understanding general bacterial activity is important to elucidate the interaction between water quality and bacterial activity in highly nutrient enriched systems in order to achieve optimal fish production. The problem of stabilizing water quality becomes especially critical for intensive fish culture systems (von Lukowicz, 1982), and in tropical arid climates, where water availability is limited and a high organic load results from fish-feeding and excretion.

In Israel, fish ponds with high stocking densities require recycling culture systems, and efficient water purification techniques. The metabolic activity of fish and microorganisms

leads to changes in the concentrations of the inorganic nitrogen compounds NH_4-N, NO_2-N and NO_3-N. If the system cannot be operated at 'steady-state' conditions, NH_4-N and NO_2-N can accumulate so as to become toxic.

The level of nitrogen in the pond water can be controlled with nitrification and denitrification. Since the efficiency of these two processes depends (among other things) on different oxygen conditions they are usually handled in two separately-operating water treatment units. Both units can be introduced in the water flow of a recycling culture system (van Rijn and Rivera, 1990). Inside these units, microorganisms, either nitrifiers or denitrifying bacteria, are involved in treatment of nitrogen compounds, while groups of organisms, heterotrophic microorganisms and several kinds of invertebrates, participate in the water purification process.

Microorganisms can 'play a positive role' in water quality control in conjunction with the remineralization and decomposition of organic matter, or the detoxification via nitrification and denitrification (cf. Jones, 1985). However, there are some negative events which often run simultaneously, e.g. ammonification and incomplete nitrification. In addition, the presence of pathogens can pose a problem (Edwards et al., 1981).

Water purification in aquaculture recycling systems has to ensure the stimulation of specific groups of bacteria, which are capable of removing metabolic products (e.g. several nitrogen compounds) that may accumulate to critical levels. To this end, knowledge is required on the distribution and the activity of all the bacteria involved in the system. Thus far, the activity of heterotrophic bacteria, nitrifiers and denitrifiers has been mainly evaluated through water chemistry, without a closer look at the microorganisms themselves. This paper is a first attempt to relate water treatment processes to basic bacteriological parameters.

Materials and Methods

The study used one experimental concrete fish basin at the Experimental station of the Ministry of Agriculture, Division of Fisheries, Ginosar, Lake Kinneret, Israel. The basin was connected

to two water treatment units. The design of these units and the culture system itself is described in detail by van Rijn and Rivera (1990).

The principle of water-flow through the system components and the investigated sample sites (A to I) are shown in Figure 1. Small quantities of system water were replaced continuously from a large underground reservoir through the inflow (A). Water was pumped from the subsurface (G), through either the biofilter (H) or the two columns of the denitrification unit (I), before leaving through each outflow back (D, E) to the surface of the basin (B). At one side of the system a water spillway was located for the outflow of the basin water (C). Sludge (F) was removed once a day by opening a valve. The outflow water was channeled back into the underground reservoir.

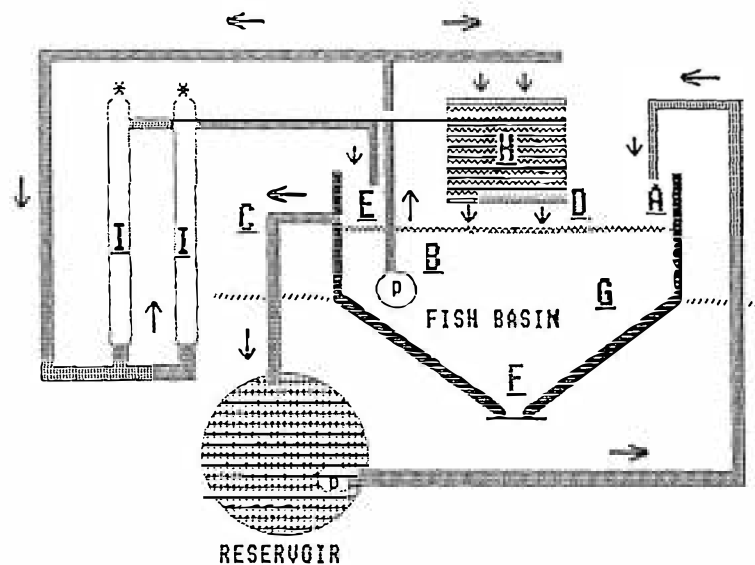

Figure 1: Schematic representation (not to scale) of the design of the semi-closed intensive fish-culture unit in Ginosar. Status: September 1989. Letters mark sample sites. A: water inflow; B: surface water; C: water outflow; D: water outflow biofilter; E: water outflow denitrification unit; F: sludge; G: subsurface water; H: biofilter, equipped with plastic material as substrate from bacterial colonization; I: denitrification unit, equipped with sand as substrate for bacterial attachment; p: pumps; *: impellers. Arrows indicate direction of water flow.

The fish basin had a water volume of 50 m^3 and was stocked with tilapia and carp at a density of 10 kg m^{-3}. Food was provided continuously from about 5:00 h to 17:00 h. Commercially-available feed pellets (15 kg day^{-1}) were used.

The distribution of bacteria was studies at nine specific selected sites (A - I), representing the relevant system components (Table I).

Table I. Sites of microbiological studies in the recycling culture system and water volume of each system unit. Positions of sample sites see Figure 1. The surface area of the plastic material within the biofilter was 400 m^2, the amount of sand in the denitrification unit about 0.026 m^3.n = number of samples taken.

	sample site	water volume (m^3)	n
A	water inflow	about 14 d^{-1}	4
B	surface water	about 10 (estimated)	26
C	water outflow	about 14 d^{-1}	28
D	outflow bio-filter	240 d^{-1}	9
E	outflow deni-trifying unit	29 d^{-1}	13
F	sludge	0.25 d^{-1}	10
G	subsurface water	about 40	4
H	interior bio-	2	4
I	interior deni-trifying unit	0.26	10

More than 100 samples altogether were taken between the end of August and the end of September 1989. These were collected at different times of the day during several 24 h cycles. Total bacteria numbers (TBN) and frequency of dividing cells (FDC) were determined in all samples by the acridine orange direct count method (Meyer-Reil 1983). Ultrasonification was used in samples which contained particles in order to remove attached bacteria. All samples were diluted prior to counting to obtain a cell density between 10 and 30 cells per microscope field. To calculate mean TBN or FDC, bacteria were counted which were visible in 40 microscope counting-grids (of 40 x 40 um^2 $grid^{-1}$). In addition, the bacteria concentration of the fish food was similarly checked.

The proportion of bacteria cells showing INT-reaction was determined in several samples, as a function of the microscopically visible formation of red formazan spots inside bacteria cells. This reaction is based on the uptake of yellow tetrazolium salt INT (2-(Iodophenyl)-3-(p-Nitrophenyl) 5-Phenyl-2H-tetrazoliumchloride) by metabolic active cells. It provides an indication on the activity within the electron transport system of the cellular respiration chain (Zimmermann et al., 1978). Samples taken from the sludge (F) or from the interior of the denitrification unit (I) were incubated with INT-concentrations ten times higher than those used by Zimmermann et al. (1978), to prevent substrate limiting effects. For all the microscopic analyses a Zeiss Axiophot epifluorescence microscope with 1250 x magnifications was used. As a result of these measurements it was possible to quantify directly the number and the activity of the bacteria population.

In addition, to analyse the population structure of the microorganisms, samples taken from the interior of the water treatment units were investigated by preparing scanning electron microscope graphs (SEM Nanolab 7, Semco). The critical point drying method was used for sample preparation.

Simultaneous to bacteria counts, water was analysed for nitrogen compounds such as NH_4-N, NO_2-N, NO_3-N (van Rijn and Rivera, 1990). Samples for this purpose were taken from the surface water (B) of the basin, from the outflow of the biofilter (D) and from the outflow of the denitrifying unit (E). Two replicates of each sample were analysed. This determination was done to test of

possible correlation between the numbers or activity of bacteria cells, and changes in concentrations of the pertained water quality criteria (e.g. nitrogen compounds).

Results

1. BACTERIA DISTRIBUTION

The total bacteria number at each sample site did not vary drastically during the day. The relative coefficient of variation varied between 2.5% and 4.4% only. Data of a typical time series from the surface water of the fish basin are shown (Table II).

Table II. Variation in total bacteria number (TBN) from the surface water (sample site B in Figure 1) at different hours (h, 20/ to 21/9/1989). The range of variation is similar to other sample sites.

h	TBN x 10^8 (ml^{-1})	± Standard deviation	Coefficient of variation (%)
06:30	1.193	0.189	2.5
10:20	1.082	0.218	3.2
14:40	1.076	0.202	3.0
18:40	0.846	0.154	2.9
22:30	1.108	0.173	2.5
02:30	1.277	0.259	3.3
06:40	1.330	0.238	2.9
10:00	1.042	0.217	3.3
15:00	0.887	0.242	4.4
18:30	1.082	0.215	3.2

The minima and maxima of TBN determinations varied at all sample sites by a factor not exceeding 4 (Table III) and this variation did not show any apparent periodicity. Only samples from the interior of the denitrifying unit (I) resulted in a larger range of values (factor of about 15, Table III).

Table III. Microbiological results from the nine sample sites within the culture system at Ginosar (compare Figure 1 for sites). Total bacteria number (TBN) expressed as mean$\pm$ standard deviation and range (in brackets). INT = percentage of bacteria cells showing respiratory activity, the INT-reaction, expressed as mean with range (in brackets). n = number of samples used for INT-analyses. All results relate to ml^{-1} except: F (ml^{-1} of sludge: a mixture of bottom water, fish faeces and unused feed), H (cm^{-2} of plastic material) and I (ml^{-1} of sand/water mixture).

	sample site	TBN (ml^{-1})	INT (%)	n
A	water inflow	$8.65 \pm 0.51 \times 10^5$ ($8.05 - 9.22 \times 10^5$)	-	4
B	surface water	$1.30 \pm 0.50 \times 10^8$ ($0.66 - 2.63 \times 10^8$)	7.5 (4.0 - 12.1)	6
C	water outflow	$5.51 \pm 1.62 \times 10^7$ ($3.39 - 9.26 \times 10^7$)	19.0 (9.4 - 33.7)	18
D	outflow bio-filter	$1.06 \pm 0.18 \times 10^8$ ($0.66 - 1.28 \times 10^8$)	13.0 (6.4 - 15.2)	7
E	outflow deni-trifying unit	$0.97 \pm 0.31 \times 10^8$ ($0.43 - 1.38 \times 10^8$)	12.2 (8.6 - 17.5)	7
F	sludge	$2.87 \pm 1.14 \times 10^9$ ($2.05 - 5.44 \times 10^9$)	13.2 (7.0 - 21.0)	8
G	Subsurface water	$4.85 \pm 0.37 \times 10^7$ ($2.90 - 10.3 \times 10^7$)	15.0 (6.4 - 20.8)	4
H	interior bio-filter	$2.82 \pm 0.76 \times 10^7$ ($1.72 - 3.28 \times 10^7$)	17.8 (14.3 - 21.4)	4
I	interior deni-trifying unit	$2.03 \pm 0.93 \times 10^{10}$ ($0.53 - 8.13 \times 10^{10}$)	11.3 (10.1 - 13.8)	6

Bacteria counts resulted in the lowest concentrations (about 10^6 bacteria ml^{-1}) in the water entering the basin at the inflow. (A, Table III). The highest number was found in the sludge (F) with about 10^9 bacteria ml^{-1} of sludge/water mixture as well as in the interior of the denitrifying unit (I), with about 10^{10} cells ml^{-1} of sand/water mixture. Bacteria number was similar in the surface water of the basin (B), in the water from the outflow of the biofilter (D) and in the outflow of the denitrifying unit (E), with about 10^8 cells ml^{-1}. Bacteria numbers were about 10^7 cells ml^{-1} in the water from the outflow of the fish basin (C), and in the subsurface water (G). Counts from the interior of the biofilter (H) gave a colonization of about 10^7 bacteria cm^{-2} of the plastic material. The number of bacteria within pellets of the fish food was about 10^6 cells g^{-1} dry weight (n = 2).

In spite of water circulation and extensive water mixing of the entire system, total bacteria number was different between each sample site. The largest differences between samples was found to be four orders of magnitude (site A versus site I, Table III).

With the SEM, different populations of bacteria could be detected in both water treatment units (Figure 2a-d). Some colonies of bacteria were found on the surface of the plastic material taken from the interior of the biofilter (Figure 2a). These colonies consisted of 20 to 40 cells, were covered with slime and fixed to the plastic material (Figure 2b). These bacteria are likely to be ammonia-oxydizers. However, they did not seem to be a dominant element of the bacteria population and did not totally cover the filter surface area.

In contrast, the surface of the sand grains taken from the interior of the denitrifying unit was covered with dense layers of different bacteria, in particular at gain spots not exposed to abrasion (Figure 2c). Here again, some bacteria were fixed with slime to the substrate. However, no colonies similar to those identified on the plastic material taken from the biofilter were encountered (Figure 2d). Unfortunately SEM-technique was insufficient to identify with certainty typical denitrifiers.

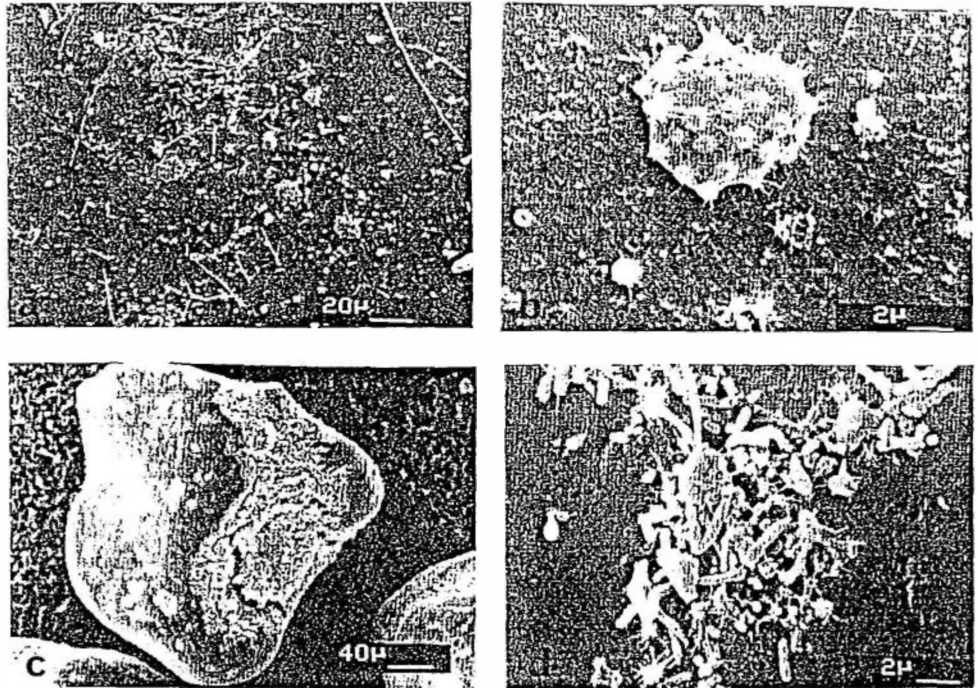

Figure 2 a-d: Comparison of the microbiological colonization of the surface of different material sampled from the interior of the water treatment systems (SEM photography).

2 a: Surface of the plastic material from the interior of the biofilter. Among many different forms of bacteria such as rods and cocci, 4 colonies are clearly visible. The surface of the plastic material is only partially covered with bacteria, possibly as a result of grazing.

2 b: Colony of bacteria, probably ammonia-oxidizing cells from the biofilter, showing fixation of cells to the plastic surface to prevent cell erosion.

2 c: Sand grain from the interior of the denitrifying unit covered with dense mats of bacteria. The surface is not completely covered with bacteria, probably because of abrasion processes during sand/water mixing inside the columns.

2 d: Accumulation of different bacteria on the surface of a sand grain from the interior of the denitrifying unit. Single cells are fixed with slime but no colonies are visible; compare the plastic material of the biofilter (2a, b).

2. BACTERIAL ACTIVITY

The percentage of frequency of dividing cells (FDC) was very low in every sample at any time of the day (sample mean between 0.2% - 4.1% of TBN). There was no marked daily variation of FDC in any of the sample sites (e.g. Table IV). Due to the very small absolute number of bacteria, counted as dividing cells, a comprehensive statistical analysis of the results was not possible.

The mean proportion of cells with visible respiratory activity (the INT-reaction) was similar for all sample sites and varied within one order of magnitude only (around 10% - 20% of TBN, Table III). A relatively high proportion of INT-active cells was found in the out-flow of the fish basin (C, mean: 19%) and inside the biofilter (H, mean: about 18%). A relatively low proportion of INT-active cells was observed in the surface water of the basin (B, mean: about 8%).

Table IV. Variation of counted bacteria (B), dividing (FDC) and respiring cells (INT-activity) expressed as means ± standard deviations prior to calculating total bacteria numbers. Samples were taken from the outflow of the fish basin (C) during different daytimes (h, 20/ to 21/9/1989). Additionally the percentage of INT-activity bacteria was calculated. Variation of data was similar to other sample sites and investigation periods.

h	B ± std.		FDC ± std.		INT ± std.		INT (%)
10:40	13.9	2.5	0.125	0.335	1.300	1.159	9.4
12:30	15.4	3.2	0.350	0.534	2.875	1.505	18.6
14:40	19.5	3.1	0.225	0.423	3.250	1.691	16.7
18:40	13.0	2.4	0.150	0.362	2.050	1.219	15.8
22:30	15.9	3.2	0.300	0.564	3.500	1.553	22.1
02:30	12.2	2.7	0.225	0.480	2.100	1.355	17.2
06:40	18.3	3.1	0.350	0.580	3.725	2.100	20.3
10:10	14.4	2.7	0.200	0.405	1.775	1.405	12.3
15:00	16.6	2.7	0.175	0.447	4.400	1.737	26.6
18:30	15.7	3.1	0.125	0.335	4.600	1.997	29.3

The variation of INT-activity was relatively small at each sample site (ranges are given in Table III) and without any obvious daily periodicity (e.g. Table IV). The lowest fluctuations of the INT-activity were found inside the two water treatment units (H, I, Table III). Minima and maxima differed by a factor of 1.5 and 1.4, respectively. At the outflow of these units (D, E) the INT-activity varied by a factor of 2.4 and 2.0, respectively. A higher variation (by a factor of 3) was found in the surface water of the basin (B) as well as in the sludge (F). In samples from the subsurface water (G) and in the outflow of the fish basin (C), INT-activity varied by a factor of 3.3 and 3.6, respectively. It was impossible to identify INT-active cells in the inflow of the fish basin (A) due to their small cell size.

3. VARIATION OF INORGANIC NITROGEN COMPOUNDS

To compare over a 24 h cycle the variation in concentrations of inorganic nitrogen compounds, daily sampling was confined to periods of over 6 hrs. This strategy followed the 12 h interval set for fish feeding. The observation periods were: morning hours (M: 5:00 h - 11:00 h), afternoon hours (A: 11:00 h - 17:00 h), evening hours (E: 17:00 h - 23:00 h) and night time (N: 23:00 h - 5:00 h).

In general, the water quality was similar at all sample sites (Table Va-c). Ammonia peaked before nitrite followed by nitrate. The total ammonia concentration was the only parameter that clearly showed daily fluctuations. Concentrations increased from the morning (0.02 - 0.46 mg NH_4-N L^{-1}) to the afternoon (0.34 - 1.75 mg NH_4-N L^{-1}) and peaked in the evening (0.54 - 2.45 mg NH_4-N L^{-1}). Thereafter, night values were reduced to 0.14 - 0.98 mg NH_4-N L^{-1} and declined further until morning hours (Table Va-c). NO_2-N concentrations were in principle fairly constant (1.0 - 3.5 mg NO_2-N L^{-1}, Table Va-c), though a slight decrease in concentration was noticeable from evening towards morning and afternoon. The amount of NO_3-N was almost constant over a 24 h cycle. At the end of September however, the NO_3-N concentration had gradually increased from 5 mg to 24 mg NO_3-N L^{-1} (Table Va-c) by a factor of around 5 compared to the initial value in early September.

Table V. Water quality characteristics in an intensive recycling culture system. Daily variation in nitrogen compounds at three sample sites within the basin: surface water (B, 5a), top; outflow of the biofilter (D, 5b), center; and outflow of the denitrifying unit (E, 5c), below. Values are expressed as means (mg/L) ± standard deviations. n = number of measurements. Intervals: morning (M: 5.00 h - 11.00 h), afternoon (A: 11.00 h - 17.00 h), evening (E: 17.00 h - 23.00 h), night (N: 23.00 h - 5.00 h).

Va) Surface water of the fish basin

B	NH_4-N mg/L	± std.	n	NO_2-N mg/L	± std.	n	NO_3-N mg/L	± std.	n	date
A	0.93	0.14	4	1.03	0.13	4	4.95	1.15	4	03-Sep-89
E	0.71	0.36	4	1.51	0.22	4	5.80		2	03-Sep-89
M	0.21	0.14	4	1.20	0.02	4	6.50	0.10	4	04-Sep-89
A	0.46	0.33	6	1.20	0.05	6	5.80	0.57	6	04-Sep-89
A	1.70	0.47	4	1.96	0.06	6	17.40	0.71	6	20-Sep-89
E	2.45		2	2.29		2	16.60		2	20-Sep-89
M	0.46	0.03	4	2.48	0.11	4	16.60	1.00	4	21-Sep-89
A	1.33	0.21	4	2.22	0.01	4	17.25	1.25	4	21-Sep-89
M	0.41		2	1.98		2	20.50		2	25-Sep-89
A	0.87	0.13	4	2.09	0.13	4	20.20	0.60	4	25-Sep-89
E	0.98	0.18	4	2.70	0.09	4	22.65	1.05	4	25-Sep-89
N	0.28		2	2.69		2	21.90		2	26-Sep-89
M	0.09	0.02	4	1.49	0.16	4	23.60	0.10	4	26-Sep-89
A	1.00		2	1.42		2	23.20		2	26-Sep-89

Vb) Outflow of the biofilter

D	NH_4-N mg/L	± std.	n	NO_2-N mg/L	± std.	n	NO_3-N mg/L	± std.	n	date
A	0.82	0.10	4	1.08	0.15	4	5.75	0.65	4	03-Sep-89
E	0.54	0.24	4	1.55	0.28	4	6.30		2	03-Sep-89
M	0.18	0.11	4	1.16	0.01	4	6.90	0.30	4	04-Sep-89
A	0.34	0.25	4	1.40	0.04	6	6.50	0.54	6	04-Sep-89
A	1.32	0.44	4	2.05	0.08	4	17.35	0.45	4	20-Sep-89
E	1.89		2	2.39		2	17.30		2	20-Sep-89
M	0.27	0.02	4	2.42	0.13	4	17.15	0.75	4	21-Sep-89
A	0.91	0.07	4	2.32	0.03	4	17.30	0.30	4	21-Sep-89
M	0.28		2	1.88		2	21.90		2	25-Sep-89
A	0.62	0.23	4	2.08	0.10	4	22.40	0.40	4	25-Sep-89
E	0.69	0.19	4	2.65	0.05	4	22.20	0.80	4	25-Sep-89
N	0.14		2	2.43		2	22.30		2	26-Sep-89
M	0.04	0.04	4	1.41	0.17	4	23.50	0.00	4	26-Sep-89
A	0.63		2	1.42		2	22.80		2	26-Sep-89

Vc) Outflow of the denitrifying unit

E	NH_4-N mg/L	± std.	n	NO_2-N mg/L	± std.	n	NO_3-N mg/L	± std.	n	date
A	0.78	0.09	8	1.21	0.15	8	6.40	0.45	8	03-Sep-89
E	0.55	0.32	8	1.69	0.27	8	6.05	0.35	4	03-Sep-89
M	0.02	0.02	4	1.10	0.00	4	7.35	0.05	4	04-Sep-89
A	0.53		2	1.68		2	6.30		2	04-Sep-89
A	1.75	0.64	8	2.12	0.10	12	16.63	0.64	12	20-Sep-89
E	2.42	0.00	4	2.39	0.05	4	16.15	0.35	4	20-Sep-89
M	0.35	0.13	6	2.69	0.14	6	16.70	1.22	6	21-Sep-89
A	1.06	0.18	4	2.44	0.05	4	15.70	1.20	4	21-Sep-89
M	0.25	0.05	4	2.07	0.06	4	20.80	0.40	4	26-Sep-89
A	0.63	0.21	8	2.42	0.31	8	20.60	0.82	8	25-Sep-89
E	1.09	0.23	6	3.45	0.55	8	18.45	2.86	8	25-Sep-89
N	0.98	0.79	4	3.36	0.40	4	20.00	1.60	4	26-Sep-89
M	0.22	0.21	8	2.06	0.91	8	21.98	1.16	8	26-Sep-89
A	0.73	0.04	4	1.60	0.00	4	21.65	0.35	4	26-Sep-89

Discussion

Nitrogen compounds such as NH_4-N and NO_2-N are harmful to fish (Colt and Armstrong, 1981). Detoxification of these compounds, which result from metabolic activity of fish and microorganisms, is necessary for an efficient water quality management in aquaculture (Boyd, 1982; von Lukowicz, 1982). NH_4-N, NO_2-N and NO_3-N can be removed from the pond water by bacteria specialized in nitrification (Jana and Roy, 1985) and denitrification (Jana and Patel, 1985). Thus far, investigations into microbiological parameters, combined with simultaneously determined water quality analyses, have seldom been documented for fish ponds. Bacteriological data have been obtained commonly by agar plate counting techniques or most probable number determination (Korzeniewski and Korzeniewska, 1982; Ram et al., 1982; Pillay et al., 1989a). Cell numbers found were 2-5 x 10^5 cells cm^{-3} of water for heterotrophic bacteria (Korzeniewski and Korzeniewska, 1982). These samples were taken from a region of water around trout culture cages. Ram et al. (1982) gave 6 x 10^6 cells g^{-1} dry weight of sediment from the number of aerobic bacteria, sampled from the bottom of a fish pond. Less than 1% of these aerobes were ammonia-oxidizing, nitrite-oxidizing and denitrifying bacteria. Pillay et al. (1989a) estimated in a marine, closed pawn-culture system concentrations of about 10^4 nitrifying bacteria ml^{-1} and about two orders of magnitude higher numbers within a sand filter being integrated into the culture system. Such culture methods allow workers to distinguish between bacteria groupings of various species. They are however, very selective and cannot include all of the species of interest. In contrast, the acridine orange direct count method used in this investigation enables the investigator to detect all bacteria present in a sample. This method furnishes the detection of higher bacteria numbers. In addition it can give an indication of the total bacterial load of a single system compounds. However, it does not enable workers to determine the truly active bacteria.

Bacteria can enter the fish basin via the water inflow (A) and via the fish food. The total bacteria number derived from these sources, about 10^6 cells ml^{-1} of water and about 10^6 cells g^{-1} dry weight of food, is low. The relatively small bacteria load in the inflow of water may be due to the low organic matter and the presence of few particles in the water coming out of the reservoir

into the basin. The number of bacteria in the fish food itself is unexpectedly low and cannot be considered as a major contributor to the overall input of bacteria.

In the subsurface water (G) however, the bacteria number had increased by one order of magnitude, maybe due to the better oxygen and food conditions in this water body. The same number of bacteria as in the subsurface water (10^7 cells ml^{-1} of water) leaves the system at the outflow of the basin (C). These two water bodies are directly connected.

The sludge (F), which is removed once a day, contains a total bacteria number of 10^9 cells ml^{-1} of sludge/water mixture. This cell density is probably caused by the utilization of the high particle number (fish faeces and unused feed settled at the base of the basin) as a substrate for colonization and nutrition. By removing the sludge every day, many bacteria leave the system. Furthermore, this same quantity of bacteria must be produced every day, which indicates a high activity in the sludge. However, that was not detectable in an unusually high proportion of dividing bacteria cells.

From the subsurface water-body, water is pumped into the two water treatment systems. Inside the biofilter (H) the plastic material is covered with bacteria but surprisingly some areas were free of colonization. This could be due to grazing.

It is also obvious from this study that ammonia-oxidizing bacteria are not the most common element of the bacteria population. By pumping the high volume of water (240 m^3 day^{-1}) together with some sludge into the biofilters, the nitrifiers and ammonia-oxidizing bacteria inside are exposed to a very high competition pressure with respect to space for colonization and oxygen for respiration. Future design criteria for a filter system should include considerations on enhanced bacterial removal from the water entering the aerobic biofilter.

Inside the columns of the denitrifying unit (I), the total bacteria number is highest of all values found in the basin (10^{10} cells ml^{-1} of sand/water mixture). This is most likely caused by the presence of densely packed, but moved by inflowing water, sand particles within the columns. These sand grains provide an

extremely large surface area for microbial colonization (cf. Meyer-Reil, 1986). An additional factor for this extreme concentration of bacteria is the high amount of sludge introduced together with the inflowing water (29 m^3 day^{-1}) into the columns. This sludge should be a carbon source necessary for bacteria, especially denitrifiers (Narkis et al., 1978). In the system investigated, an internal carbon source (the sludge) was used instead of external sources as is usual (Balderston and Sieburth, 1976). However, the results from water quality analyses yielded several times inconsistent interpretations, especially for processes at the outflow of the denitrifying unit. These phenomena were discussed in more detail in the paper of van Rijn and Rivera (1990).

On the surface of sand grains, in the interior of the columns of the denitrifying unit, an accumulation of different bacteria occurred. Although most probably not dominant, some denitrifying bacteria were present. Denitrifiers could be isolated (van Rijn and Rivera, 1990), and several times water quality data gave indications of denitrification (van Rijn and Rivera, 1990, this study). To establish conditions favorable for the growth of denitrifiers, the pH oxygen concentration and substrate availability should be manipulated inside the denitrifying unit, depending on the special situation just present. In modern systems design-criteria will have to provide means to allow for such manipulation.

The water leaving the two treatment units (D, E) contained about the same number of bacteria (10^8 cells ml^{-1} of water). This similarity could have been caused by the intensive mixing of the subsurface water (10^7 bacteria ml^{-1}) and the sludge (10^9 bacteria ml^{-1}) entering both units. From the outflow of each unit (D, E) the water was released to the surface of the fish basin (B), which therefore had the same TBN.

No obvious daily periodicity in total bacteria number was observed at any of the sample sites. Total bacteria numbers with a difference of at least one order of magnitude can doubtlessly represent different situations of a bacteria population (Meyer Reil, 1983, 1986). Adding fish-food indirectly changed the amount of NH_4-N in the basin via excretion of fish. But neither the fish food itself nor the varying NH_4-N concentration had a drastic

effect on the total bacteria number.

There was also no apparent periodicity in the frequency of dividing bacteria cells. The percentage of these cells was unexpectedly low, given that the addition of fresh food, in the form of commercial feed pellets, would be likely to increase the dividing activity of bacteria. Besides methodical problems (low number of analysed microscope counting grids), the reasons for this low percentage may be a rapid division, together with a rapid separation of cells, due to the high water temperature (28°C). On the other hand, a constant rate of division at all sample sites may have been established, which could indicate balanced growth. Additionally, bacteria grazers such as protozoa (cf. Sherr and Sherr, 1984) can maintain bacteria populations at a discrete level. Peritrich ciliates were discovered in the interior of both water treatment units, colonizing the surface of the plastic material and of the sand grains. It may be that protozoa or small invertebrate grazing liberates space on the surface of substrates.

The second indication of bacterial activity, the percentage of INT-reactive cells, was nearly constant (within the same order of magnitude) at each sample site over 24 hrs. Again, no influence on INT-activity was visible by adding fish food or by the variation of NH_4-N concentration. Expressed as absolute numbers, the amount of INT-active cells was nearly the same for the sample sites B, C, D, E and G, where the water was mixed intensively. Expressed relatively, in percentage, the mean of INT-activity for the surface water (B) was lower by a factor of about 2 compared with the other sites. Perturbation of the surface of the fish basin, caused by aeration with the help of a paddle-wheel or the inhibitory influence of the sun's radiation to bacteria (Diab and Shilo, 1988) could be a reason for this relatively low INT-activity.

Best conditions in INT-activity of bacteria cells seemed to be given inside the biofilter (H), and in the water at the outflow of the fish basin, possibly resulting from bacteria washed out of the organic material which had settled at the base. Because active microbial cells are present in outflow leaving aquaculture systems, this water should be examined microbiologically more often (cf. Hejkal et al., 1983).

In general, however, most of the counted bacteria cells in the system showed no INT-activity in spite of the potentially good food supply and a sufficient amount of oxygen (5 mg L^{-1}).

Results of INT, FDC or TBN analyses of the same order of magnitude are well known from other biotopes. Zimmermann et al. (1978) investigated brackish and freshwater samples, using epifluorescence microscopy together with INT-activity measurements. They found between 5% and 35% respiring bacteria of total bacteria numbers. This is similar to results from this study. Shares of FDC varied between 2.0% and 5.0% of the total bacteria population in sediments analysed by Meyer-Reil (1983), sampled from an 18-m station located in the Western Kiel Bight (Baltic Sea, Germany). The results from this study give possible FDC values of < 5%. Meyer-Reil (1986) found numbers of bacterial colonization in marine sandy sediments very close to those data presented from the denitrifying unit of the fish pond investigated.

To balance input and output of bacteria for the fish basin in Ginosar, 63 times more bacteria must leave the system per day at point (C) than enter at point (A) (mean of TBN x water volume m^3 day^{-1}). Apparently there is a very high daily production of bacteria cells in the basin, but due to the dynamic processes and the intensive water mixing within the system, the bacteria production cannot be detected directly in drastic variations of TBN, or in expected high shares of FDC. Possible changes of specific bacteria groups would be more easily detected at the investigated sample sites.

Assisted by scanning electron microscope, some morphological details of the bacteria and their technique of attachment to the substrate were made visible (cf. Dempsey, 1981). Weise and Rheinheimer (1978) have described the bacterial colonization of sand grains from a brackish water area. They found similar techniques of attachment for bacteria and similar places of bacterial colonization on the surface of sand grains. For the identification of nitrifiers, however, which is important for the potential performance of a biofilter to be calculated, transmission electron microscopy (Johnson and Sieburth, 1976; Pillay et al., 1989b) or immunofluorescence methods (Fliermans et al., 1974) are mandatory.

Towards the end of the investigation the biofilter performed well during the whole day. The decrease of NH_4-N varied between 29% and 56% during the different investigation periods, calculated for different daytimes, comparing the outflow of the biofilter and the surface water of the fish basin (Table Va, b). No drastic change in concentrations of NO_2-N was observed between these two sample sites. The biofilter did not seem to be a location where the main increase in NO_2-N took place.

However, at the outflow of the denitrifying unit the NO_2-N concentration increased substantially compared with the surface water of the basin (especially at 25/ to 26/9/89, Table Va, c). This could be an indication of decomposition of organic matter or of incomplete nitrification within the columns.

Several times, the amount of NO_3-N showed a considerable increase at the outflow of the biofilter, where complete oxidation of NH_4-N to NO_3-N should occur (3/ to 4/9/89 or morning and afternoon of 25/9/89, Table Va, b). However, at the outflow water of the denitrifying unit, where a decrease of NO_3-N would be expected, results were inconclusive (decrease at 20/ to 21/9/89 or 26/9/89 and 25/9 evening; increase at 3/ to 4/9/89, Table Va, c). It is assumed that the denitrifying unit was not working efficiently, although the intention of the original system design was to enhance sufficiently the anaerobic denitrification (van Rijn and Rivera, 1990).

Recommendations for future system-design are to stabilize the anaerobic conditions for an optimal function of the denitrification unit and to prevent, inside this unit, decomposition of organic matter to nitrite. Additionally, the carbon supply plus the availability of carbon for bacteria in this unit should be improved. To get a higher performance from the biofilter, the number or the activity of nitrite-oxidizing bacteria should be enhanced. This could be done by reducing the organic load entering the filter together with a reduction of the number of heterotrophic bacteria. These culture systems still require more detailed knowledge about the composition of the bacteria population (including fish- and human-pathogenic bacteria), about the productivity of bacteria which are important for the nitrogen-cycle during the running system and about the bacteriological composition or activity of the water leaving the

culture unit. This will lead to a better understanding of conditions favourable for bacteria involved in water purification processes for recycling systems. The realization of this knowledge will ultimately protect public health and the environment as well as optimize productivity in aquaculture.

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