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Pathways of bacterial contamination during egg incubation and larval rearing of turbot, *Scophthalmus maximus*

by

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ABSTRACT

Averall microbial levels in the water of a turbot farm were similar to those in regular seawater, except in Artemia cultures and in turbot egg incubation jars at the end of the incubation period, where bacteria counts were one or two orders of magnitude higher. The numbers of colony forming units (CFU), which can be considered as potential pathogens, were up to four orders of magnitude higher in the Artemia cultures and egg incubation jars than in the rest of the hatchery facilities. Rinsing of the food organisms (rotifers) prior to feeding them to turbot larvae, however, significantly reduced bacterial numbers, thus reducing the level of cross-contamination. Several species of Aeromonas, Pseudomonas and Vibrio, among others, were identified in the eggs and larvae, or in the water in which these were incubated and cultured. Scanning electron microscopy showed that the surface of unfertilized turbot eggs is a breeding ground for bacteria; therefore, the early removal of these eggs from incubation jars is recommended. The epidermis of turbot larvae was virtually free of microorganisms.

INTRODUCTION

The hatchery production of turbot (Scophthalmus maximus, L.) is faced with two major difficulties: adequate nutrition and good water quality (Kirk, 1979; Quantz et al., 1988). While the problem on improving the quality of feeds for turbot larvae has gained increasing attention by scientists, the influence of water quality upon survival rate of the larvae remains to be investigated more thoroughly, not only considering conventional water quality criteria but also bacterial contamination.

Water quality depends to a large extent on the biochemical activities of the microorganisms found within the water mass of the culture unit (Kawai et al., 1964). Microbial proliferation may cause epizootic outbreaks of disease leading to mass mortalities, particularly during the sensitive juvenile stages of fish (Shotts and Bullock, 1975; Nellen, 1983; Nicolas et al., 1989). High fish population densities and high feeding rates in commercial aquaculture units generally promote a rapid multiplication of microbial populations. Microbial colonization of fish eggs has been

investigated by Hansen and Olafsen (1989) in cod (Gadus morhua)) and halibut (Hippoglossus hippoglossus), but similar studies on other species seem to be lacking (Sauter et al., 1987a).

The purpose of the present study was to estimate the bacterial populations associated with the hatchery culture of turbot, and to identify problem areas. To achieve this goal, total bacterial counts and the determination of colony forming units (saprophytes, including potentially pathogenic bacteria; Rheinheimer, 1981) were determined on eggs and larvae and in the hatchery water system. Major species groups of bacteria were identified.

MATERIAL AND METHODS

Location

Samples were taken from June to October, 1990, at the facilities of BUTT Company which are located at Strande near Kiel, Federal Republic of Germany.

Hatchery design

The farm operates with seawater from the outer Kiel fjord of 15 to 22 ppt salinity (generally referred to as "brackish water" throughout the text), which is pumped from the Baltic Sea and passed without sterilization through a series of storage tanks, sand, and cartridge filters.

System layout is depicted in Figure 1 showing the various relatively independant culture units of the hatchery: water supply system with its pre-treatment units and the brackish water distribution system to the fice major components of the entire hatchery operation as well as the effluent discharge system. Water supply to the culture tanks runs from the central header unit to separate lines, including the food chain unit (algal tanks, Artemia tanks, Brachionus cultures) and to the incubation and rearing units. Only the latter two are operated under continuous flow conditions for most of the time (except for the first 2 days after fertilization).

Operational Procedures

Fertilized turbot eggs were mass incubated in brackish water at 14°C in previously formaldehyde-sterilized 4 L jars at a density of 12,000 eggs per L. The water was renewed at at constant flowthrough rate of 6 L/min. After 4 days, the eggs were transferred to formaldehyde-sterilized 1800 L tanks, in which the temperature gradually increased to 20°C during the weeks of study. Most larvae hatched at day 5. Beginning on day 7, they were initially fed twice daily with rotifers (Brachionus plicatilis), and after about 2 weeks with Artemia nauplii (data on the aomint of food given to the turbot larvae are not available). Water was not exchanged in the nursery tanks until 22 days after fertilization. Thereafter, sea water was supplied at a rate of 20L/h.

Rotifers were cultured in formaldehyde-sterilized 80 L tanks and fed with algal cultures (Nannochloris sp.) grown in 150 L polyethylene bags. Before being fed to turbot larvae, rotifers were rinsed on a 200 µm mesh sieve with filtered brackish water.

Artemia eggs were disinfected for 30 min in a 20 ppm hypochlorite solution and incubated at 28°C in formaldehyde-sterilized 60 L tanks at a density of 0.7 g/L. The nauplii generally hatched after 2 days. After hatching they were fed with "unsaturated fatyy acids" (Artemia Systems NV-SA; Ghent, Belgium), harvested two hours after feeding, rinsed with filtered sea water and fed to the turbot larvae.

Sampling and sample treatment

For the analysis of bacteria on turbot eggs, two or three jars were sampled daily in each of three 4-days series. In the case of the turbot larvae, two tanks were sampled at 3-day intervals in both of thw series lasting 24 days. Artemia cultures were sampled daily during their 2-day incubation period; two culture series were studied: one with 5 ppm of a disinfectant (Actomar B 100; Ciba-Geigy) and one with untreated sea water. Other points in the water (indicated in Figure 1) were sampled periodically, in order to obtain reference data on the overall bacterial load of the culture system.

For total bacteria counts on the surfaces of turbot eggs and larvae, these were rinsed with sterilized brackish water, fixed with formaldehyde at the culture site(3% final concentration), and then transported to the laboratory. For the identification of bacterial species, about 300 egs or 5 larvae, respectively, were rinsed with 500 ml of sterile brackish water on a sterile sieve and homogenized with 1ml of sterile brackish water and sterile glass beads (2mm diameter) for three minutes in a homogenizer (Vibrogen; Edmund Bühler, Tübingen, Germany). A dilution series up to 10-6 was immediately prepared with sterile brackis water. From each dilution, 100µl were spread on agar plates for microbiological analysis (see below) and the plates were incubated for 14 days at 25°C in an incubator. Each sample was inoculated in duplicate.

Water samples were taken with a sterile pipette at several locations of the culture system as shown in Figure 1, and transferred to sterile 100 ml glass bottles. Two 100 µl subsamples from each bottle were spread on agar in the same dilutions used for the egg and larval homogenates, and incubated in the same way. The remainder of each water sample was fixed with formaldehyde (3% final concentration) and brought to the laboratory for determination of total bacteria counts (see below). Water samples were taken also from the algal and rotifer cultures, but bacterial levels could not be determined with accuracy, because of the interference with algal cells or yeat cells which were fed to the rotifers. Microbial populations on tank walls and water pipes were not investigated.

Microbiological laboratory analysis

Turbot eggs were characterized as "viable" or "unviable" after observation under an optical microscope ("unviable" eggs were mostly unfertilized or lacked an embryo). Eggs and larvae surfaces were examined for bacterial growth with a digital scanning electron microscope (Zeiss DSM 940), after dehydration in ethanol, critical point drying and coating with gold-palladium. The scans of the surface sections (area = 505 μm^2) were printed with a videographic printer. Depending on the intensity of bacterial colonization of the egg surfaces, between 5 and 40 sections from each egg were evaluated by counting all bacteria on the print. The results were extrapolated to give the total number of bacteria per egg (estimated surface = 2.3 mm^2, or 4570 sections). The count was stopped when the number of

bacteria on one section exceeded 5000 (corresponding to 23 x 10^6 bacteria per egg), since precise estimates were impossible at such a high bacterial population density.
Numbers of total bacteria counts in the water samples were achieved according to the method of Zimmermann and Meyer-Reil (1974), modified by Hobbie et al. (1977). The samples were diluted if necessary, 1 ml subsamples were filtered through black nuclepore filters (pore size: 0.2 µm), and dyed with aqueous acridine orange solution (0,2 g/L). The bacteria of 40 grids were counted under the epifluorescence microscope.

Colony forming units were determined on ZoBell brackish water (ZB) agar (Rheinheimer, 1977). Homogenized larvae and eggs were inoculated on tryptosesoy-agar (TSA, Caso; Merck Art. 5458), which is recommended particularly for fish pathogenic bacteria. Haemolytic activity was tested on blood agar (Merck Art. 10886). The presence of *Vibrfo* was tested on TCBS agar (Merck Art. 10263). For the identification of bacterial species colonies were picked at random from ZB agar and cultivated to obtain pure cultures. These were identified by gramstaining, and observing their morphology and motility under a standard microscope. Cytochrome oxydase was determined according to Kovacs (1956). Catalase was determined with 3% hydrogen peroxide (Wang and Fung, 1986). Gram-negative rods were identified with the API 20E test kit (Api Systems, Montalieu Vercieu, France) using sterile brackish water as the suspending medium.

RESULTS

The total bacteria counts and colony forming units throughout the farm's water system were similar to those found *in si~u* in the Baltic (Table 1). In the *Artemia* tanks, the bacteria counts, especially saprophytes, increased drastically after the nauplii were fed with emulsified fatty acids. Treatment with the antibiotic reduced total bacteria only marginally and had no effect on colony forming units (data in Table 1 are pooled). Rinsing with brackish water, however, virtually eliminated the input of external bacteria from the *Artemia* culture into the turbot nursery tanks. This has been confirmed by microbiological analyses at the farm (Quantz, pers.comm., 1992), and SEM studies, which showed the surface of rinsed *Artemia* to contain almost no bacteria.

The total bacteria counts in the water of the egg incubation jars did not increase noticably until the 3rd or 4th day after fertilization. Colony forming units, however, multiplied more rapidly than total bacteria counts, and increased continuously during incubation (Table 1). In the larval nursery tanks, the total bacteria counts increased by about one order of magnitude, and the CFU increased by about two orders of magnitude during the first days of nursery culture. This increase coincidented with the initial temperature increase from 13°C to 20°C in the nursery tanks and the time of onset of feeding with rotifers. After water exchange had been started on day 18 of the incubation of the nursery culture, the influent and effluent water contained less bacteria than the tanks themselves (Table 1, bottom).

Only few bacteria were observed on viable eggs until the last day of incubation (Table 2; Figs. 2a+3), whereas unviable eggs were rapidly colonized (Table 2; Figs. 2b and 4). Heavily colonized eggs often had secondary layers of bacteria growing over the first layer (Figs. 4c and 4d). The numbers of bacteria on the epidermis of the larvae were too low to be quantified (Fig. 5). Identified species of gram-negative rods found in association with turbot eggs and larvae are listed in Table 3.

DISCUSSION

The passage of the Baltic water through the sedimentation and storage tanks and through the various filters, as well as sterilization of the tanks used for the cultivation of turbot and their food organisms prior to their use in a rearing series, apparently lead to a slight overall decrease in the initial bacterial numbers, as compared to natural seawater use. This did not seem to hold true, however, for potential pathogens. Whenever culture conditions led to a general increase in bacterial levels, saprophyte proliferation was disproportionately higher. Identified gram-negative bacteria associated with the turbot eggs and larvae (Table 3) are also representative for adult flatfish (Liston, 1957). The list includes several potential pathogens, such as *Aeromonas hydrophila* and *Vibrio* spp., which may cause mass mortalities in fish (including turbot) hatcheries (e.g. Bullock et al., 1965-Horne et al., 1977; Austin, 1982; Egidius, 1987). Nicolas et al. (1989) found that Vibrio spp. represented 70% of the bacteria found in turbot larvae in rearing systems.

There are various causes for bacterial growth on and in fish eggs, such as primary colonization of dead sperm (Rosenthal and Odense, 1986) or maternal infection of the eggs (Evelyn et al., 1984; Sauter et al., 1987b; Hansen and Olafsen, 1989). In the present study, the surface of viable turbot eggs was not heavily colonized by bacteria until the fourth day of incubation. This was preceeded by microbial proliferation on the unviable eggs, on which bacteria began to grow in several layers by day 3, and led to total loss of an egg charge in one of the three investigated incubation series. It is probable that the strong increase of bacterial levels in the water on the last day of incubation is due to the dead eggs' role as a breeding ground for bacteria. This may explain why various methods, such as UV-treatment of the influent water, sterilization of egg surfaces (Evelyn et al., 1984) and the use of probiotics (Gatesoupe, 1991) are often unsuccessful to cope with the problem. Although the use of antibiotics can be effective in killing these bacteria, their use may create new problems in the long run (e.g. selecting for resistant strains). It is suggested that lower incubations densities and repeated removal of dead (i.e. deformed) eggs should be the most effective methods to minimize the infection risk for healthy eggs. A general reduction of the initial microbial counts is important, because even heavy colonization of the egg surface with non-pathogenic bacteria can be detrimental to egg development by changing gas transfer properties of the egg membrane and thereby causing hypoxia in high density batches much earlier than would be expected in non-commercial, small-scale laboratory trials. (Hansen and Olafsen, 1989).

Bacterial levels in the water of the larval tanks were comparable to those in natural seawater (Table 1). A slight increase during the first week of nursery culture corresponded to the concomitant increase in water temperature and the beginning of feeding with rotifers. The lower counts in the effluent water after the beginning of flowthrough may have been an effect of the 200 µm filters at the exit of the tanks. These filters retained faeces and dead feed organisms.

From the results of the present study, it appears unlikely that bacteria in the water of the rotifer and *Artemia* cultures, or on the surface of these food organisms represent a major problem in turbot nursery culture, provided they are rinsed well with filtered water before being fed to the larvae. On the other hand, rotifers and *Artemia* can ingest large quantities of microorganisms which may be passed along in the food chain to turbot larvae. This problem was investigated in detail by Nicolas et al.

(1989). The use of antibiotics or probiotics in rotifer and *Artemia* cultures may attenuate detrimental effects from associated bacteria (Perez Benavente & Gatesoupe, 1988; Gatesoupe, 1991), but further studies are needed in order to obtain solutions on a commercial scale.

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Table 1: Total bacteria counts and colony forming units of different water samples taken at the turbot hatchery farm. Locations of sampling points within the system of the farm are marked (crosses) in Fig. 1. ~ = average data for Baltic Sea water were taken from Zimmermann (1977; Table 10.3, St. 2) and Rheinheimer (1977; Table 11.2, St. 2). N = number of samples taken over a period of 2 months.

Location	Total bacteria (10^6/ml) Median	Min	Max	Colony forming units (10^3/ml) Median	Min	Max	N
Baltic Sea water (Kiel, outer fjord)	2.5	0.74	6.0	5.8	1	33	
Storage tank	1.1	0.50	1.6	2.0	1	160	4
Header tank	0.9		1.9	1.0	0	71	19
After Filters	0.6		1.6	2.0	0	68	21
After small storage tank	0.6		2.4	1.0	0	100	27
Artemia cultures							
before rinsing	38.0	34.00	61.0	27,000.0	19000	38000	7
after rinsing	2.7	2.50	2.9	970.0	930	1000	2
Egg incubation jars							
day 0	0.5	0.40	3.3	1.5	0.05	2	8
day 2	1.0	0.50	3.2	71.0	21	2000	8
day 4	16.0	6.00	48.0	2900.0	1800	4300	6
Larval rearing tanks							6
water inside tanks: day 0-6	1,4	0.20	2.4	0.7	0.2	60	
day 9-5	2.5	2.00	5.7	84.0	58	220	6
day 18-24	1.7	0.70	2.6	77.0	14	210	6
Influent water day 18-24	0.1	0.07	0.2	0.06	0.01	0.08	6
Effluent water day 18-24	0.6	0.10	0.5	20.0	15	37	6

Table 2. Total bacteria counts (10^6/egg) on the surface of turbot (Scophthalmus maximus L.) eggs during the four day incubation period. N(e) = number of eggs counted; N(s) 0 number of sections (videographic prints) counted

	Viable eggs total bacteria counts ($x10^6$/ egg)					unviable eggs total bacteria counts ($x10^6$/ egg				
Day	Median	Min	Max	N(e)	N(s)	Median	Min	Max	N(e)	N(s)
0	0	0	0	60	900	-	-	-	-	-
1	0	0	0	25	850	0	0	4.0	30	800
2	0.6	0.4	0.9	15	100	0.9	0.7	1.1	30	250
3	0.2	0.09	0.5	15	100	>23	>23	>23	15	75
4	3.7	2.4	>23	15	75	>23	1.7	>23	30	150

Table 3. Species of gram-negative bacteria isolated from turbot (Scophthalmus maximus) eggs and larvae, and from the water in which these were incubated or reared.

Species	Egg incubation jars	Turbot eggs	Larval nursery tanks	Turbot larvae
Acinobacter calcoacetivus	+	+		
Aeromonas hydrophila		+	+	+
Aeromonas sobria			+	
Acromobacter spp.		+		
Enterococci			+	
Flavobacterium multivorum	+			
Moraxella spp.	+	+		
Pseudomonas aeruginosa		+		
Pseudomonas fluorescens	+	+	+	+
Pseudomonas putida				+
Pseudomonas sp.		+		
Pseudomonas vesicularis			+	
Vibrio parahaemolyticus		+	+	
Vibrio fluvialis			+	

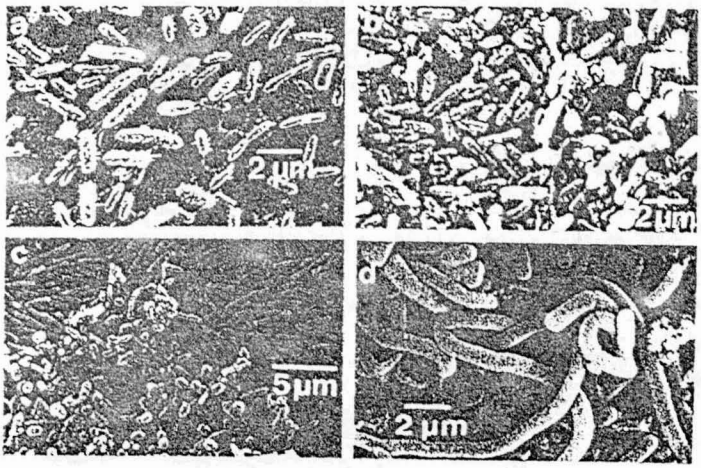

Figure 4: Surface of moribund (or unfirtilized) turbot eggs. (a) after one day of incubation (numerous bacteria are attached by trheads to the egg surface); (b) after two days (a second layer of bacteria is beginning to form); (c) after three days (the primary layer of bacteria is covered by an extensive secondary layer; (d) after four days (a dense biofilm covers the egg surface)

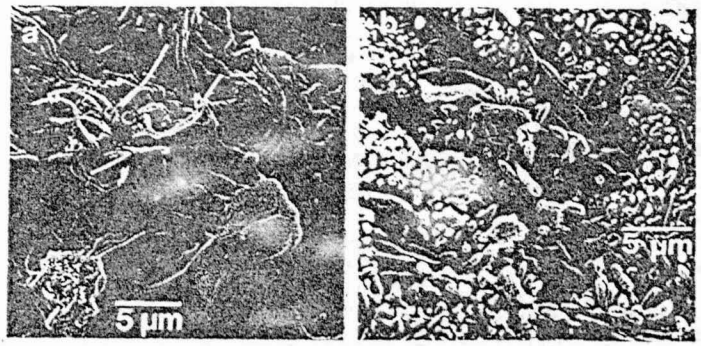

Figure 5: Scanning electron micrograph from the epidermis of turbot larvae. (a) 9 days after hatching (a single bacterium is on the lower right corner; the filamented field (center) is a neuromast); (b) = 12 days after hatching (epidermis covered by an unusually large number of bacteria)

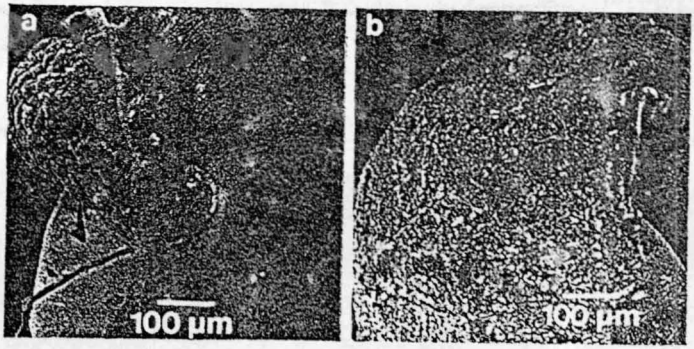

Figure 2: Turbot *(Scophthalmus maximus)* eggs after four days of incubation. (a) healthy (normal) embryo; begin of hatching (head just breaks through the egg envelope); (b) dead egg covered by a continuous bacterial biofilm

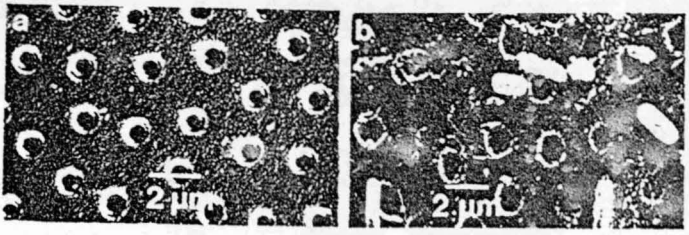

Figure 3: Surface of viable turbot eggs: (a) after one day of incubation. (egg surface showing membrane pores free of bacteria); (b) after three days of incubation (some bacteria scattered on egg surface)

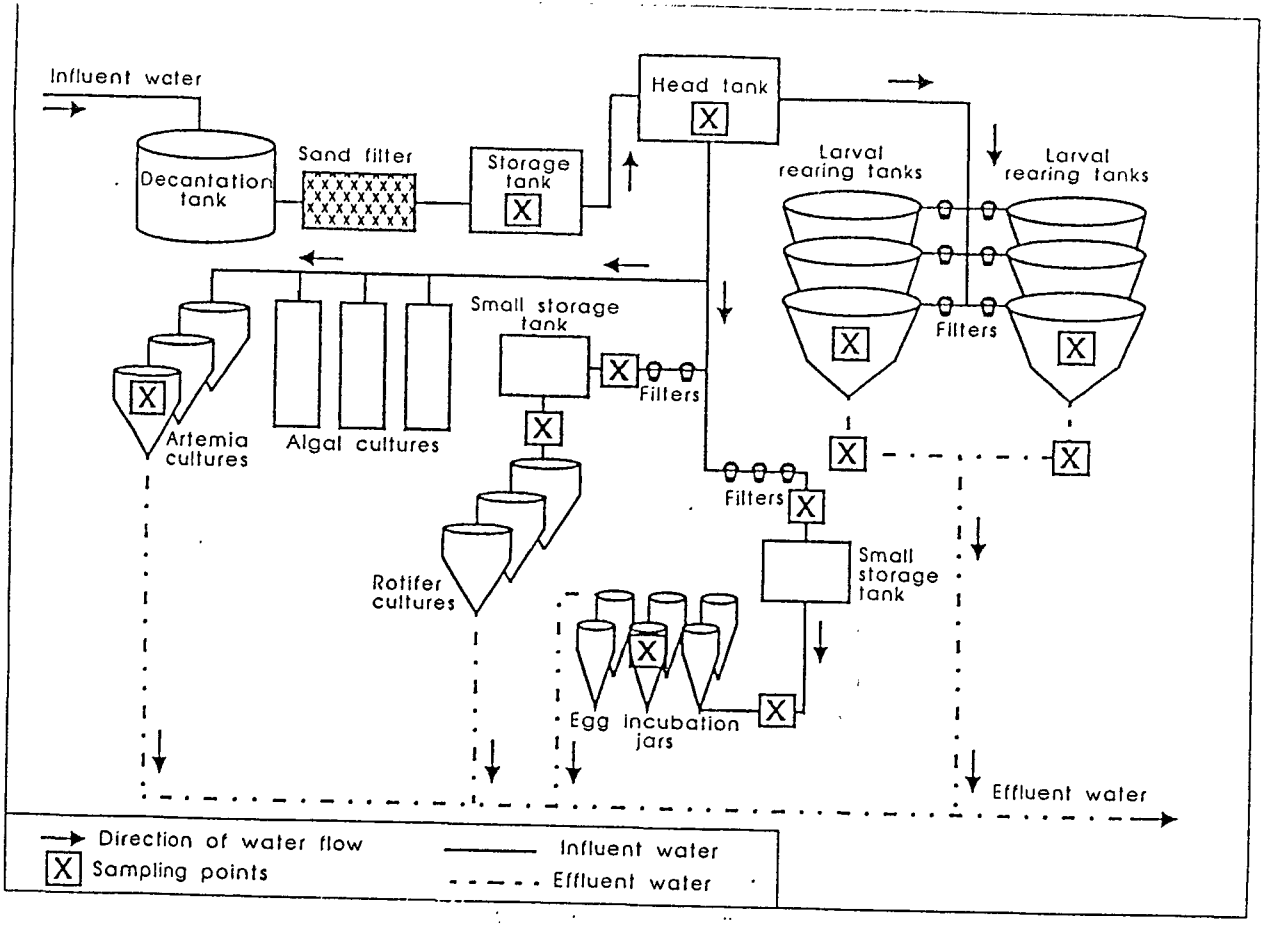

Figure 1: Flow-diagram of the water supply and discharge system for all system components in the turbot hatchery; arrows indicate direction of water flow; x = sampling points