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TECHNICAL NOTE

A method for the early assessment of the quality of turbot larvae

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The bacterial environment should be taken into account for assessing the quality of fish larvae, besides biochemical and developmental criteria. Detrimental effects of associated flora have been observed on the survival rates of larvae (e.g. Nicolas *et al.*, 1989), and experimental infections were performed on fish larvae by introducing the pathogen either directly into the rearing medium or into live food organisms (e.g. Kusuda *et al.*, 1986). A challenge test was used for assessing the effect of a food additive on the ability of turbot (*Scophthalmus maximus*) larvae to resist infection, but that required rearing the larvae separately (Gatesoupe, 1993). Bergh *et al.* (1992) have proposed an infection test with transfer of eggs into polystyrene dishes, but it was not convenient for larvae with exogenous food source. The present paper was an attempt to develop a new test with transfer of larvae after feeding. Then this test was inserted into a simple survey of the quality of turbot larvae, with a view to drawing up relations between rearing success, associated bacteria and resistance against pathogens.

For this purpose, seven batches of larvae were successively reared in 150 l cylindrical tanks with conical bottoms from day 1 after hatching until day 10. Three tanks were used for batches 1, 3, 5 and 6, while five tanks were used for batches 4 and 7. The larvae of batch 2 were reared in one tank. The rearing temperature was gradually increased from 14 °C to 18 °C by day 5. The salinity was 35%. For feeding the larvae, rotifers (*Brachionus plicatilis*) were cultured in seawater according to the method already described (Gatesoupe, 1990). The rotifer diet was made up of baker's years, menhaden (*Brevoortia tyrannus*) oil (Sigma F8020), soybean lecithin, DL-α-tocopherol (USB), and vitamin premix (Spyridakis *et al.*, 1988), at the daily rates of 60.8, 7.7, 0.7, 0.015, and 0.7 mg l^{-1}, respectively, on a dry matter basis. The larvae were fed with the rotifers from day 3 to day 9, at the daily rates of 20, 50, 75, 100, 150, 200, and 250 rotifers per initial larva, corresponding to the seven consecutive days of feeding. Each day, the rotifers were transferred from their culture medium into clean seawater tanks (200 rotifers ml^{-1}), where they were enriched with spray-dried fish autolysate, menhaden oil, soybean lecithin, and DL-α-tocopherol, at the rates of 15, 7.7, 0.7, and 0.015 mg l^{-1}, respectively, on a dry matter basis. The rotifers were continuously distributed to turbot with a peristaltic pump, the flow rate of which was adjusted so that the whole volume was carried through in 24 h. At day 10, the survivors were counted and 30 fish were sampled in each tank,

fixed in a 5% formalin–seawater solution, then weighed 1 month later, because of the fast loss of weight during this first month of storage (Lockwood, 1973).

Bacteria were counted at day 8 in five larvae sampled per replicate. The samples were handled under sterile conditions, while using half-salinity water (18%; aliquot volumes of distilled and seawater) for all the operations. They were first rinsed over a 180 μm net, then they were suspended and crushed in 4.5 ml of water in a glass homogenizer. Three consecutive tenfold dilutions of the suspension were performed, so as to inoculate 1 ml of the 10^{-3} dilution into a Petrifilm Aerobic Count Plate 6400 (3M Health Care; containing Plate Count Agar), and 0.1 ml of the 10^{-2} and 10^{-3} dilutions into plates with Thiosulphate–Citrate–Bile Salt agar (TCBS Cholera medium, AES Laboratoire) dissolved in half-salinity water (18%). The colonies were counted on the Petrifilm and TCBS plates after minimum incubation times of 4 and 2 days, respectively, at 20 °C.

The resistance of turbot against a pathogenic vibrio was challenged at day 9. The pathogenic strain corresponded to that previously described as '*Aeromonas hydrophila*' (Gatesoupe, 1991) according to its Api 20 E profile (Bio Mérieux). However, it should be better designated as *Vibrio* sp., because it was sensitive to 0/129 (2,4-diamino-6,7-di-*iso*propyl-pteridine, Diagnostics Pasteur), while it required NaCl for growth (Santos *et al.*, 1993). Considering that the strain was arginine-dihydrolase-positive, lysine-decarboxylase-negative, and ornithine-decarboxylase-negative, it would belong to the third group described by Alsina and Blanch (1994), but this strain could not be further identified with the keys proposed by these authors. In this study it was called vibrio 'P', and cultivated on half-salinity TCBS agar. A second strain was cultivated on the same medium as a control bacterium for the challenge test. According to its biochemical characteristics, it would belong to the same third group as vibrio P. It differed from the pathogen in six Api 20 E tests (i.e. it was β-galactosidase-, indole-, Voges–Proskauer-, gelatinase-, and mannitol-negative, but sucrose-positive). It was isolated from apparently healthy turbot larvae, but they were contaminated by high amounts of this single strain. Any mass mortality observed with turbot larvae was never related to this vibrio, which was designated as vibrio 'NP'. The NP colonies were yellow on TCBS agar, whereas the P colonies were green. The vibrios were incubated at 20 °C on half-salinity TCBS agar, 1 day before inoculation. The inoculum concentration was adjusted by the transmission (%) of 570 nm light through the suspension, before pouring 1 ml per 500 ml polycarbonate square bottle (Nalgene) used for the challenge. Then the concentration of the suspension was counted on half-salinity TCBS plates incubated for 24 h at 20 °C. There was a difference of light transmission through the two suspensions, and the counts of vibrio P inoculated per bottle were between 10^8 and 10^9 colony forming units (CFU), whereas they were only 10^7–10^8 CFU with vibrio NP. For each replicate, one bottle was used as a control without adding any bacterium, while another one was inoculated with vibrio NP, except for batch 2 where these two treatments were triplicated. Vibrio P was inoculated into duplicates for each tank of batches 1, 6 and 7, triplicates for batch 2, and only one bottle per tank of batches 3, 4 and 5. Fifty larvae were gently introduced into each bottle, then kept in 500 ml of seawater at 18 °C. After 48 h of incubation, the survivors were counted through the transparent wall of the bottles, without opening them. Then, the bottles were sterilized by autoclave or chlorination.

The results obtained with the 23 tanks were compared by analysis of variance. Eight variables were considered, i.e. survival rates and mean weight at day 10, bacterial counts in 8 day-old larvae on Petrifilm, TCBS agar, differentiating between yellow and green

TABLE 1. Mean rearing results of the 23 tanks of turbot from seven batches (average with 95% confidence interval for mean). The batch effect was assessed by analysis of variance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant). The larvae were counted and sampled for weighing at day 10, while bacteria were counted in 8 day-old turbot, and the challenge test was performed at day 9

	Average	Confidence interval	Batch effect
Survival (%)	66	4	***
Weight (mg)	0.57	0.03	***
CFU larva^{-1} on			
Petrlfim	11×10^4	2×10^4	**
TCBS-yellow	17×10^3	4×10^3	*
TCBS-green	2×10^4	3×10^4	n.s.
Challenge test survival (%)			
Control	80	3	***
Vibrio NP	82	2	***
Vibrio P	46	3	***

colonies, and survival rates of the challenge test at day 9, with either vibrio NP or vibrio P, or without vibrio. Significant differences among batches were observed with all the variables but TCBS green colonies (Table 1). The survival rate of 10-day-old turbot was low for batch 2 (30%), and high for batch 5 (95%). The minimum mean weight was observed also in batch 2 (0.37 mg), whereas the maximum corresponded to batch 4 (0.70 mg). The Petrifilm counts varied from 2×10^4 (batch 1) 2×10^5 CFU larva^{-1} (batch 7), while the TCBS yellow colonies varied from 3×10^3 to 3×10^4 CFU larva^{-1} for the same batches, respectively. The survival rate of the control group of the challenge test was high, except in batch 2 (0%), and in batch 3 (69%). The same observation could be noted in the group challenged with vibrio NP, with survival rates of 0.3% and 81% in batches 2 and 3, respectively. The survival rate of turbot challenged with vibrio P varied from 0.7% (batch 2) to 85% (batch 6). Then significant partial correlations were investigated among the eight variables. When several bottles per replicate were tested with the same challenge treatment, their mean survival rate was considered for correlation study. Only four partial correlations were found significant. The most significant was the correlation observed between the survival rates of the control group without vibrio, and that of the group challenged with NP (correlation coefficient $r = 0.93$). This latter survival rate was also correlated to the survival rate at day 10, observed in normal rearing condition ($r = 0.57$). A negative correlation was observed between the survival of the challenge control group and the amount of green colonies growing on TCBS agar ($r = -0.55$). Lastly, there was a positive correlation between the survival rate of the group challenged against vibrio P and the amount of colonies counted on Petrifilm ($r = 0.51$).

The variability of the quality of the seven batches is illustrated by the variability of the results obtained. When the classical criteria are considered – i.e. survival rate, mean weight, and bacterial counts – no correlation can be drawn between them. Only the challenge test may be related to these criteria, and it could reveal hidden quality aspects. The simple transfer of the larvae into seawater bottles may be considered as an 'activity test' (also called 'handling test', Kitajima *et al.*, 1979), though the larvae were not netted

out from water, but gently poured into bottles. It is important to keep such a control group in the challenge test, so as to distinguish the mortality due to handling from that due to infection. For instance, the resistance to infection could not be observed in batch 2, the quality of which was obviously bad, while a mortality peak was observed within 24 h in this case. It seems possible to use this control group as an indicator of less obvious flaws of the quality, as in batch 3, which looked good, except that the survival rate of the control group was low. The survival rates obtained either with vibrio NP or without vibrio are similar, and this vibrio seems harmless for turbot in these conditions. The positive relation found between the survival rate observed after 48 h of challenge with this vibrio and that obtained in normal rearing conditions is not interesting in practice, and this treatment could be discarded from the challenge test. However, the challenge with vibrio P seems sensitive, because the survival rates were different from batch to batch. It is important to adjust the initial concentration of the inoculum with a view to obtaining the mortality peak within 48 h, so as to avoid a longer stay of the starved larvae in ageing water, while giving them a chance to resist infection. The dose lethal to 50% of the larvae within 48 h seems convenient. It could not be estimated precisely due to batch effect, but it may correspond to a 1 ml inoculum with approximately 60–70% of light transmitted at 570 nm, which gave an initial concentration of about 10^6 CFU ml^{-1} after dilution into a 500 ml bottle. The positive relation between the resistance of larvae against vibrio P and their amount of bacteria retrieved on Petrifilm is an indication that the infection may be hindered by numerous harmless flora. In contrast, a high amount of bacteria different from vibrio P, but growing in green colonies on TCBS agar, might be detrimental to the survival rate of the challenge control. For instance, their high level (10^5 CFU $larva^{-1}$) was related to the mortality observed in the control group of batch 3. However, such considerations about associated bacteria must not be generalized, due to their high variability (Robin *et al.*, 1986). At least, it should be kept in mind that a Petrifilm count as high as 2×10^5 CFU $larva^{-1}$ is not necessarily an indication of poor quality.

In conclusion, the challenge test could be used a matter of routine for assessing within 48 h the effect of various environmental and nutritional factors on the resistance of larvae against bacterial infection, while a control group without infection must be kept as an 'activity' test.

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