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Aquaculture Research
September 2013, Volume 44, Issue 10, Pages 1511-1523
http://dx.doi.org/10.1111/j.1365-2109.2012.03158.x
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A spatiotemporal study of bacterial community profiles associated with Atlantic bluefin tuna larvae, Thunnus thynnus L., in three Mediterranean hatcheries

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Abstract :

During the first 2 years of larval rearing trials with Atlantic bluefin tuna, survival was a challenging issue. As bacterial colonization of the gut has been shown to play a key role for other species, we studied the profiles of the microbiota associated with individual larvae at different stages in three distant hatcheries. The Bacterial Community Profile (BCP) was quantified based on PCR-DGGE analyses of partial amplicons from 16S rDNA. Considerable individual variability in BCP was observed before onset of feeding, and the BCP did not show regular fluctuation during ontogenesis. Microalgae were added to the rearing tanks in two of the three hatcheries, but it was not possible to distinguish the effect of location from the effect of algal addition on BCP. In one hatchery, the larvae were reared either with algal addition or in mesocosm, but due to high individual variability, no significant difference in BCP was detected between the two groups. It was hypothesized that this variability was caused by differences in health, physiological status and developmental stage of the larvae. A practical conclusion from the study is the need to analyse a considerable number of individuals to reflect statistically significant differences between the microbial communities associated with rearing groups.

Keywords : larval rearing; bacterial diversity; DGGE; *Thunnus thynnus*

1. Introduction

Due to the collapse of bluefin tuna recruitment in western Atlantic Ocean (Safina & Klinger, 2008), the effort to develop methods for reproduction in captivity was initiated (Mylonas, de La Gándara, Corriero & Belmonte Ríos, 2010). The first attempts of larval rearing were recently published with the conclusion that further research on environmental factors and nutritional requirements are required to improve survival at early stages (de la Gándara, Mylonas, Covès, Ortega, Bridges, Belmonte Ríos, Vassallo-Agius, Papandroulakis, Rosenfeld, Tandler, Medina, Demetrio, Corriero, Fauvel, Falcon, Sveinsvoll, Ghysen, Deguara & Gordin, 2010; de Metrio, Bridges, Mylonas, Caggiano, Deflorio, Santamaria, Zupa, Pousis, Vassallo-Agius, Gordin & Corriero, 2010). Makridis, Papandroulakis, Sarropoulou & Divanach (2011) observed proliferation of bacteria associated to moribund tuna larvae. It is essential to document bacterial colonization in this new species, as microbiota-host interaction may develop into parasitic infection in fish larvae, and thus cause mortality (Olafsen, 2001; Vadstein, Mo & Bergh 2004).

The recent studies on microbial community structure in fish are based on culture-independent methods, but deal mainly with cold-water species. For example, the Bacterial Community Profile (BCP) associated with Atlantic halibut was distinct from that of the surrounding water, but with similarities between larvae sampled in different geographical regions (Jensen, Øvreås, Bergh & Torsvik, 2004). The succession of BCP was similar in batches of larvae sampled over several years, despite variable BCP in the live feeds (Plante, Pernet, Hache, Ritchie, Ji & McIntosh, 2007; McIntosh, Ji, Forward, Puvanendran, Boyce & Ritchie, 2008). However, feeds and other factors influenced BCP associated with fish larvae in other studies (Bjornsdottir, Karadottir, Johannsdottir, Thorarinsdottir, Smaradottir, Sigurgisladottir & Gudmundsdottir, 2010; Lauzon, Gudmundsdottir, Petursdottir, Reynisson, Steinarsson, Oddgeirsson, Bjornsdottir & Gudmundsdottir, 2010). This is complicated further by the fact that BCP varied almost as much between individuals from the same tank as between different hatcheries and rearing systems for cod larvae (Fjellheim, Playfoot, Skjermo & Vadstein, 2007; Fjellheim, Playfoot, Skjermo & Vadstein, 2011).

There is less data available on BCP based on culture-independent methods for temperate and warm water marine fish larvae, even though microbial management is considered essential also for Mediterranean hatcheries (Dimitroglou, Merrifield, Carnevali, Picchietti, Avella, Daniels, Güroy & Davies, 2011). The relatively high temperature required by tuna larvae may exacerbate bacterial growth in the larval rearing tanks (Nakagawa, Eguchi & Miyashita, 2007). The addition of microalgae to the rearing tanks may regulate the bacterial community, as in the “green water” (Stottrup, Gravningen & Norsker, 1995) and the “pseudo green water” method, based on the control of light intensity, photophase and feed supply, with a daily addition of microalgae to maintaining constant their concentration in the rearing system (Papandroulakis, Divanach & Kentouri, 2002). An alternative to intensive rearing systems is the mesocosm approach where large tanks are colonized by wild plankton populations (Van der Meeren & Naess, 1993). In this system, bacteria proliferate after the first phytoplankton bloom, and then remain at a constant level (Pitta, Giannakourou, Divanach & Kentouri, 1998). The mesocom approach can be combined with regular addition of cultured feed (microalgae and rotifers) to increase productivity (Divanach & Kentouri, 2000).

One goal of the SELFDOTT project – “SELF-sustained aquaculture and DOmestication of *Thunnus thynnus*” – was to compare three rearing methods in common use in Mediterranean hatcheries; clear or pseudo green water in intensive rearing, or the mesocosm approach (de la Gándara *et al.*, 2010). In this framework, it was important to study to what extent these different rearing techniques affected the colonization of individual tuna larvae by bacteria. Microbiota associated with tuna larvae were sampled at different stages in the three hatcheries during two consecutive years, with the

objective to compare the bacterial community profiles in individuals sampled within and among the rearing groups. The BCP was characterized by PCR-DGGE (Denaturing Gradient Gel Electrophoresis), and intended to evaluate the stochasticity of bacterial colonization in tuna larvae (Fjelheim *et al.* 2011).

2. Materials and Methods

2.1. Larval rearing and sampling

The eggs were obtained from ABFT broodstock of Caladeros del Mediterráneo (S.A., Cartagena, Spain), and split in three and transported to the different hatcheries. One part was incubated at IEO (Puerto de Mazarrón, Spain; Ghysen, Schuster, Covès, de la Gándara, Papandroulakis & Ortega, 2010), and the larvae were reared in pseudo green water in the local facilities. A second part of the eggs were and shipped to Ifremer (Palavas, France), where the larvae were reared in clear water. The last third was sent to HCMR (Heraklion, Greece), where the larvae were reared in two conditions – pseudo green water and mesocosm. The initial numbers of larvae are reported in Table1. The pseudo green water techniques and the mesocosm system differed mainly in the initial concentration of larvae, tank size, illumination, and water quality and dynamics. The pseudo green water techniques differed between Mazarrón and Heraklion in terms of algal supply, feed sequence and enrichment.
The PCR-DGGE analysis was done on individual larvae, which came from Mazarrón and Palavas in 2009, and from all three hatcheries in 2010. Larvae were collected at 2 or 3 dph (day post hatch), and at 7, 10 or 12 dph. The mean lengths of the larvae and the size of the samples are reported on Table 2. Two batches of larvae were sampled at 3 dph in Palavas in 2009. In 2010, larvae were sampled at 3 and 15 dph in Mazarrón and Palavas. In addition, two larvae were sampled at 20 dph at Mazarrón in 2010. All the larvae were fixed immediately in RNA*later* (Applied Biosystems, Courtaboeuf, France), and then stored at –20°C.

2.2. DNA extraction

A method of DNA extraction adapted after Godon, Zumstein, Dabert, Habouzit & Moletta (1997) secured enough bacterial DNA from individual tuna larvae even at 2-3 dph. All chemicals were of molecular biology grade and were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The individuals were picked up from the RNA*later* solution, and distributed to 2ml Eppendorf tubes with 250 µl of inhibiting buffer (4 M guanidine thiocyanate, 0.1 M Tris, pH 7.5). The samples were homogenized with a dispersing aggregate unit, and incubated at 70°C for 1 h in extraction buffer (40 µl of 10% N-lauroyl sarcosine, plus 500 µl of 5% N-lauroyl sarcosine, 0.1 M phosphate buffer, pH 8.0). After cooling, the tubes were vortexed at maximum speed for 10 min with 250 µl of acid-washed silica beads (0.1-mm diameter). PCR inhibitors were removed with 15 mg Polyvinylpolypyrrolidone. The supernatant was retained after centrifugation at 18,000 g for 3 min, and the pellet was washed three times with 500 µl of buffer (50 mM Tris pH 8, 20 mM EDTA pH 8, 100 mM NaCl, 1% polyvinylpolypyrrolidone). The pooled supernatants were centrifuged again at 18,000 g for 3 min, and split in two. After addition of 1 ml isopropanol to each 1-ml fraction and centrifugation at 18,000 g for 90 min, the pellets were re-suspended in 225 µl of 100 mM phosphate buffer (pH 8) and 25 µl of 5 M potassium acetate. After centrifuging again at 18,000 g for 30 min, the supernatants from these two centrifugations were

pooled and incubated at 37°C for 30 min with 2 µl of RNase (10 mg ml^{-1}). After precipitation in 1 ml of absolute ethanol and 50 µl of 3 M sodium acetate, the DNA pellet was recovered by centrifugation at 18,000 g for 30 min. The pellet was washed twice with 75% ethanol, and once with absolute ethanol (5 min centrifugation), let dry, and re-suspended in 50 µl of autoclaved 'Mili-Q' water. The DNA concentration and the purity were estimated with NanoDrop (Wilmington, DE, USA). If the total DNA concentration was less than 200 ng μl^{-1}, the sample was treated with OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA.).

2.3. PCR of 16S rDNA, and DGGE

It was possible to obtain amplification by using universal bacterial primers in the presence of high amounts of eukaryotic DNA in the samples, with a method of nested PCR adapted from Bakke, de Schryver, Boon & Vadstein (2011). When total DNA concentration was less than 200 ng μl^{-1}, 8 µl of sample was introduced into the 10 µl of total PCR mix for the first round of the nested PCR. When there was more than 200 ng DNA μl^{-1} in the extract, 1 µl of sample was added to the PCR mix, which was completed by 7 µl H_2O. The PCR mix contained Taq DNA polymerase (0.025 U μl^{-1}), 0.2 mM of each dNTP, 0.4 µM of each primer (EUB-8-f; 984-r), and 2 mM $MgCl_2$ (initial denaturation at 95°C for 5 min; 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 48°C, and 1 min elongation at 72°C; final extension at 72 °C for 10 min). The second round restricted the amplicon to the variable region V3, with a GC clamp added for DGGE (ca. 180 bp; primers 338-f-GC and 518-r). 3 µl of PCR product from the first round was added to the PCR mix (30 µl in total; initial denaturation at 95°C for 5 min; 30 cycles with 30 s at 95°C, 30 s at 53°C, and 1 min 72°C; final extension at 72°C for 30 min).
Twenty-five µl of PCR product were loaded onto a 8% polyacrylamide gel prepared from 40% (w/v) acrylamide/N, N'-methylenebisacrylamide (37.5:1). The denaturing gradient was prepared by mixing 35 and 60% solution of 7 M urea and 40% formamide with the gradient maker provided with INGENYphorU (Ingeny International, Goes, The Netherlands). Fourteen gels were successively prepared to compare two or more groups on the same gel. The compared groups differed by either the age of the larvae, the batch, the hatchery, the rearing system, or the year of sampling (Gels I-XIV, Table 3). The electrophoreses were run for 18 h at 80V and 60°C in the phorU system, filled with 1X TAE buffer. However, only Gels I and XIV were cast in the phorU cassette. The 'sandwich core' unit of DCode (Bio-Rad, Marnes-la-Coquette, France) was preferred to improve linear migration on the other gels. The gels were stained for 30 min in 200 ml of TAE solution with SYBR Green I (0.1 µl ml^{-1}). After rinsing, the band pattern was visualized by scanner (Typhoon 9400, Amersham Biosciences, Uppsala, Sweden).

2.4. Gel image analysis and statistics

The images of the 14 gels were analysed with GelCompar II (version 6.1, Applied Maths, Sint-Martens-Latem, Belgium) for band alignment and quantification of peak areas. The percent normalized data were further analysed with the Paleontological Statistics Software Package (PAST; Hammer, Harper & Ryan, 2001). Pairwise Bray-Curtis similarities were calculated between lanes on each gel. This index is robust and well suited for relative data (Field, Clarke & Warwick, 1982; Anderson, Crist, Chase, Vellend, Inouye, Freestone, Sanders, Cornell, Comita, Davies, Harrison, Kraft, Stegen & Swenson, 2011). The Bray-Curtis similarities within and between the groups of samples were compared by one-way Non-Parametric Multivariate ANalysis Of VAriance (NPMANOVA; Anderson, 2001). For each sample the diversity indices were computed based on band pattern. When a sample was analysed several times on the 14 gels, the inter-gel average was calculated. These averages were used for

comparisons of diversity between samplings using ANOVA for cases where the requirement of normality and equality of variance were met, and with the Kruskal-Wallis test when these requirements were not met. Pairwise comparisons were done with Tukey or Dunn's test, respectively. When only two groups were compared, the Student t or Mann-Whitney test was used.

3. Results

The intra-group median Bray-Curtis similarity in the microbiota of individual larvae are presented in Table 3, and statistical analysis based on Bray-Curtis similarities between the hatcheries, the developmental stage of larvae, and the year of sampling are reported in Tables 4, 5 and 6, respectively.

The median Bray-Curtis similarity of one group varied between different gels (Table 3). This was partly due to the fact that different gels were represented by different individuals, and partly due to gel-to-gel variability. Medians around 0.3 were observed for the larval groups with the lowest intra-group similarities, indicating a large individual variability within these groups. The highest intra-group similarities were mainly observed at mouth opening in 2009 in Mazarrón (0.80 in Gels I and II, and 0.87 and in Gel IX) whereas the medians were more variable in Palavas (0.42-0.76). In 2010 the medians at mouth opening were lower in Mazarrón, compared with the groups from the two other hatcheries (0.32-0.48 in Mazarrón, versus 0.60-0.72 in the two others). After onset of feeding the median similarities within groups were more variable and in general lower than at mouth opening.

The variability between groups was mostly higher than the variability within groups. In fact significant differences were observed for all the gels except Gel XII (NPMANOVA, Table 3). This latter gel compared samples collected from the pseudo green water and mesocosm systems at 10 dph in Heraklion. The lack of significant difference in the microbiota between these two rearing systems was confirmed on Gels IV, V, and XI by pairwise Bonferroni multiple comparison (P=1, 0.19 and 1, respectively). This was due to very different DGGE band patterns of individuals within the same group, resulting in low intra-group similarity (e.g. Gel XI, Fig. 1).

When comparing Bray-Curtis similarity between hatcheries within the same gel, significant differences were found for all 9 possible comparisons at the same developmental stage (Table 4).

The groups collected at two different stages in the same hatchery and in the same year had significantly different BCP, except at Mazarrón (Gels I and VI; Table 5). However, only two individuals were sampled at 20 dph, and thus a reliable conclusion about BCP is not possible for this developmental stage.

Significant differences in BCP were detected between years for the larvae collected at Palavas and Mazarrón (Table 6). At Palavas a significant difference was detected at mouth opening between the two experimental groups in 2009. In Mazarrón the BCP of the group sampled at 12 dph in 2009 was significantly different from those of both groups sampled in 2010 at 10 and 15 dph (Fig. 2).

The individual variations in bacterial diversity indices, and statistical comparisons between groups at the same age are presented in Table 7. At mouth opening the mean dominance index was higher at Palavas in 2009 compared to the samples from 2010 at Palavas and Mazarrón. The band richness was higher at Palavas in 2010 compared with the second batch of 2009 and with Mazarrón in 2009. In these two hatcheries the Shannon's entropy in 2009 was lower than in 2010. The greatest difference in evenness was observed between the two years at Palavas. Comparisons of diversity indices in 2010 at 10 dph revealed that the mean dominance at Palavas was higher than at Heraklion, where no significant difference was found between the mean diversity indices of the two rearing systems. The band richness and the Shannon's entropy at Heraklion were low compared to the two other hatcheries. At 15 dph in 2010

there were significant differences between Palavas and Mazarrón in dominance, band richness, and Shannon's entropy.
The mean dominance, band richness and Shannon's entropy differed between the stages of development at Mazarrón in 2010, but this was due to the fact that only two individuals were sampled at 20 dph. These two larvae had lower diversity than larvae sampled at earlier stages (Fig. 3). In Heraklion, the band richness at mouth opening was higher than at 10 dph. At Palavas in 2010, the evenness was higher at 15 dph compared with the earlier samples.

4. Discussion

We restricted deliberately the comparison of the similarity in DGGE band profiles to intra-gel comparisons, as the inter-gel comparisons may result in biased conclusions (Nakatsu, 2007). However, inter-gel comparison was possible for the diversity indices, but there were not general trends of fluctuation between hatcheries, developmental stage, or year of sampling. Øvreås (2000) recommended the variable V3 region as especially suitable for the analysis of bacterial diversity by DGGE, due to its high resolution. However, the short nucleotide sequence is not optimal for phylogenetic identification, and only some well-isolated bands can be excised from the gels for re-amplification and sequencing. The identification of the bacterial community associated with tuna larvae based on other methods is in progress within the frame of the SELFDOTT project. A first screening revealed dominance by *Vibrionaceae* and *Rhodobacteriaceae* in the microbiota associated with the larvae of *Sarda sarda* (bonito), another *Scombridae*, reared under the same conditions as *Thunnus thynnus* (Makridis *et al*., 2011).
The high individual variability of microbiota associated with tuna larvae seemed in good agreement with that observed in cod larvae by Fjellheim *et al.* (2011). In this previous study, which compared feeding larvae sampled between179 and 216 days-degrees post hatch, the similarity between microbiota of individuals from the same rearing environment was comparable to the similarity between three cod hatcheries where different rearing methods were used. These days-degrees corresponded to 7-10 dph in the present rearing conditions of tuna larvae (c. 24°C). At this stage, the mean Shannon diversity index of the bacterial communities associated with tuna was between 1.9 and 2.6, which is in the same range as the mean indices estimated by Fjellheim *et al.* (2011) based on T-RFLP analysis of the bacterial community of cod larvae (1.7-2.3).
Unexpectedly, high diversity and individual variability appeared soon after mouth opening, when the larvae had not started exogenous feeding. The present results did not reveal any specificity in the microbiota initially colonizing tuna larvae. However, our data were obtained from the first rearing trials with this species, and with high mortality 2-3 days after mouth opening (Papandroulakis, Ortega, Covès, Vassallo Agius, Tandler, Stefanakis, Viguri, Vidal, Papadakis, de la Gándara, Ruelle, Anastasiadis, Mylonas & Divanach, 2010). The high variability in the microbiota of individuals might be due to variable physiological status and was possibly influenced by the stochastic inoculation due to the active uptake of bacteria by larvae (Reitan, Natvik & Vadstein, 1998).
Many reports were published on the initial colonization of fish larvae by bacteria, but very few compared results from individuals. High similarity was observed among microbiota of individuals of halibut at start feeding (Jensen *et al.*, 2004), whereas Fjellheim *et al.* (2011) concluded the opposite for cod larvae as noted above. In the other studies, where several larvae were pooled to describe the bacterial community, the conclusion arose that the bacterial community composition was highly diverse in yolk sac larvae of halibut, cod, and haddock (Plante *et al.*, 2007; McIntosh *et al.*, 2008; Bjornsdottir, Johannsdottir, Coe, Smaradottir, Agustsson, Sigurgisladottir &

Gudmundsdottir, 2009) and with differences between tanks and hatcheries (Griffiths, Melville, Cook, Vincent, St. Pierre & Lanteigne, 2001; Verner-Jeffreys, Shields, Bricknell & Birkbeck, 2003).

McIntosh *et al.* (2008) noted that microbiota associated with cod larvae underwent a rapid change after hatching, which was unique for each tank, but the succession in the microbiota after start feeding appeared reproducible in two consecutive years. Plante *et al.* (2007) reported a similar repeatability in the succession of the microbiota in many batches of larvae reared in the same hatchery over six years. No such regular succession was observed in the preliminary results on tuna larvae. Technical improvements of the larval rearing were done between the two years of sampling, and that may account at least partly for the inter-annual differences observed in the present study. Further sampling should be planned in the forthcoming years. The data from 20 dph onwards may be worth comparing with those of earlier stages. If the observed trend with a development towards a more uniform microbiota can be confirmed, it would be in accordance with observations from other species (Plante *et al.*, 2007; McIntosh *et al.*, 2008).

The introduction of yolk sac sea bream larvae as live prey from 18 dph onwards might be the reason for the apparent shift in microbiota observed at 20 dph. Most authors agreed that bacteria associated with live feed affected gut microbiota in fish larvae, but to various extents. For example, changes in microbiota occurred when halibut and cod larvae started to feed on rotifers (Verner-Jeffreys *et al.*, 2003; Brunvold, Sandaa, Mikkelsen, Welde, Bleie & Bergh, 2007; McIntosh *et al.*, 2008). However, the gut microbiota of these larvae did not reflect the bacterial community associated with the rotifers, or the highly variable microbiota of *Artemia* (Bjornsdottir *et al.*, 2009; Plante *et al.*, 2007). The bacterial community composition associated with live feed was not characterized in the present experiments, but it is likely that the individual variability observed in the microbiota of tuna larvae reflected partly what they ate. Different feeding sequences and enrichments were applied to the tuna larvae in the three hatcheries, and this might have contributed to the differences observed in the microbiota from the three locations.

The effect of rearing techniques on the bacterial community composition of the larvae was beyond the scope of this preliminary study, which aimed simply to study individual variability of the bacterial community profiles within and between different batches of larvae. The only possible comparison of rearing techniques was that of mesocosm and the pseudo green water method in Heraklion. The main difference between the techniques was the concentration of the larvae and the size of the rearing tanks, which might affect physiology and behaviour of larvae (Sloman & Baron, 2010). However, no significant effect of rearing condition was detected. This could be due to the high individual variability within treatments and limited number of individuals analysed. Other types of treatments of Atlantic tuna larvae, like probiotics, resulted in significant changes in the bacterial balance, but further studies are needed to confirm these observations (Covès, de Vogué, Desbruyères, Dhormes, Fievet, Huelvan, Lallement, Le Gall, Ruelle, Vidal, Castex, Mazurais, Cahu & Gatesoupe, 2011).

It is not possible to generalize our findings for Atlantic tuna from the limited set of experiments with sub-optimally reared larvae of this new species. However, it is clear that the microbiota associated with fish larvae fluctuated considerably among individuals, even when they were reared in the same tank. The present results confirm the previous findings of Fjelheim *et al.* (2011), and a main conclusion was that representative samples should contain many individuals. The numbers of larvae included in the samples are not always reported in the literature, and it is quite variable. For example, one to five larvae were sampled each time by Verner-Jeffreys *et al.* (2003) and Jensen *et al.* (2004). Plante *et al.* (2007) and McIntosh *et al.* (2008) used a sample size of ten larvae per sample, whereas Romero & Navarrete (2006) used 20 individuals of coho salmon fingerlings per sample. Pools of few individuals could lead to erroneous conclusions, and the optimal size of the samples should be estimated in future experiments. Analyses of individuals are recommended as long as the reasons

for the individual variability are not sufficiently understood. This individual variability reflects the complex interactions between bacteria and the larvae, even at an early stage, and the factors that regulate the colonization of the gut require further research.

Acknowledgments

This work was carried out with financial support from the Commission of the European Communities, specific RTD programme of Framework Programme 7, SELFDOTT, "From capture based to self-sustained aquaculture and domestication of bluefin tuna, *Thunnus thynnus*" (GA 212797) coordinated by F. de la Gándara. It does not necessarily reflect the Commission's views and in no way anticipates its future policy in this area. The first and the penultimate authors are also indebted to their colleagues of the ongoing FP7 project PROMICROBE, especially I. Bakke, N. Boon, P. Bossier, P. de Schryver, K. Dierckens, C. Giatsis, and J. Morillo Perez for fruitful discussions about DGGE methodology and microbial diversity in other fish larvae.

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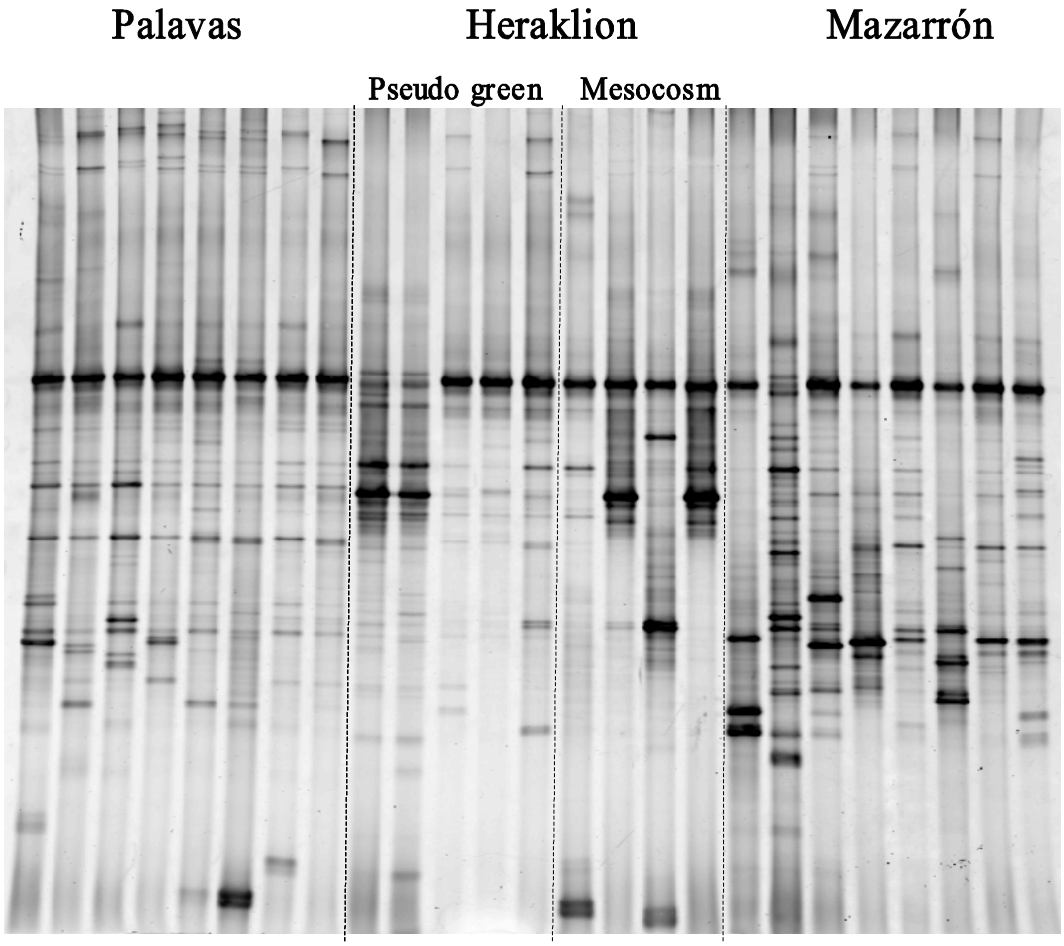

Figure 1 : DGGE profiles illustrating the individual variability observed in the bacterial community associated with individual tuna larvae sampled at 10 dph in the three hatcheries - with two different rearing methods in Heraklion. Each lane corresponded to one larva (Gel XI).

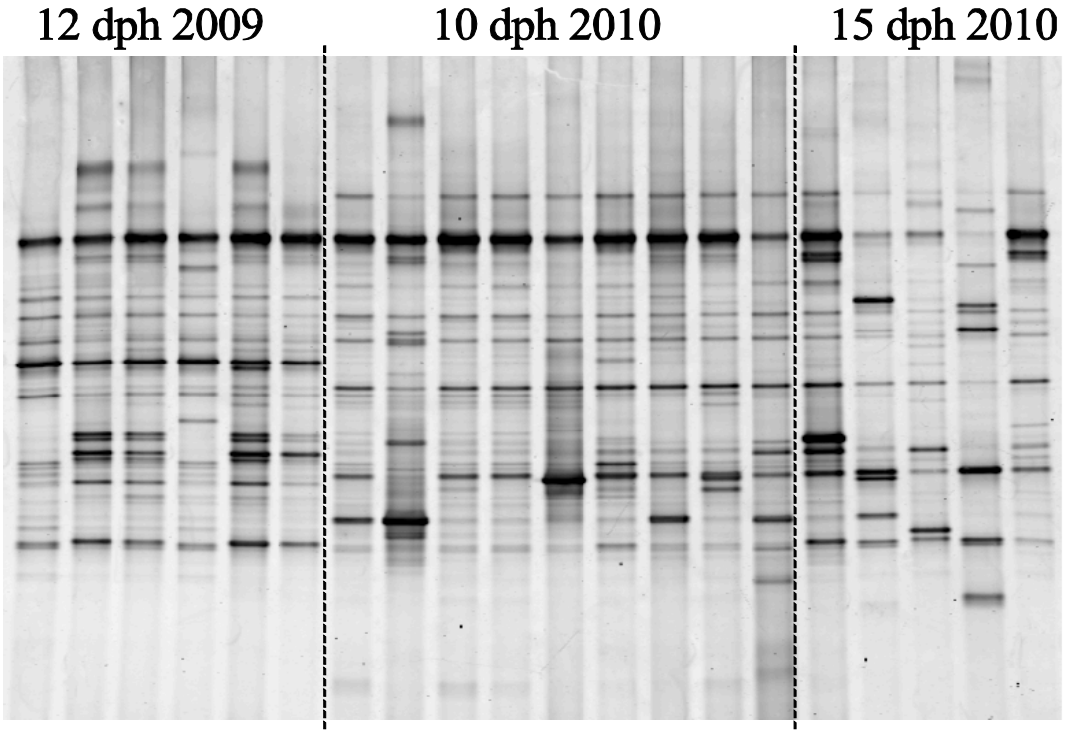

Figure 2 : DGGE profiles illustrating the individual variability observed in the bacterial community associated with individual tuna larvae sampled in two subsequent years in Mazarrón during the period of feeding on rotifers (12 dph in 2009, 10 and 15 dph in 2010). Each lane corresponded to one larva (Gel VII).

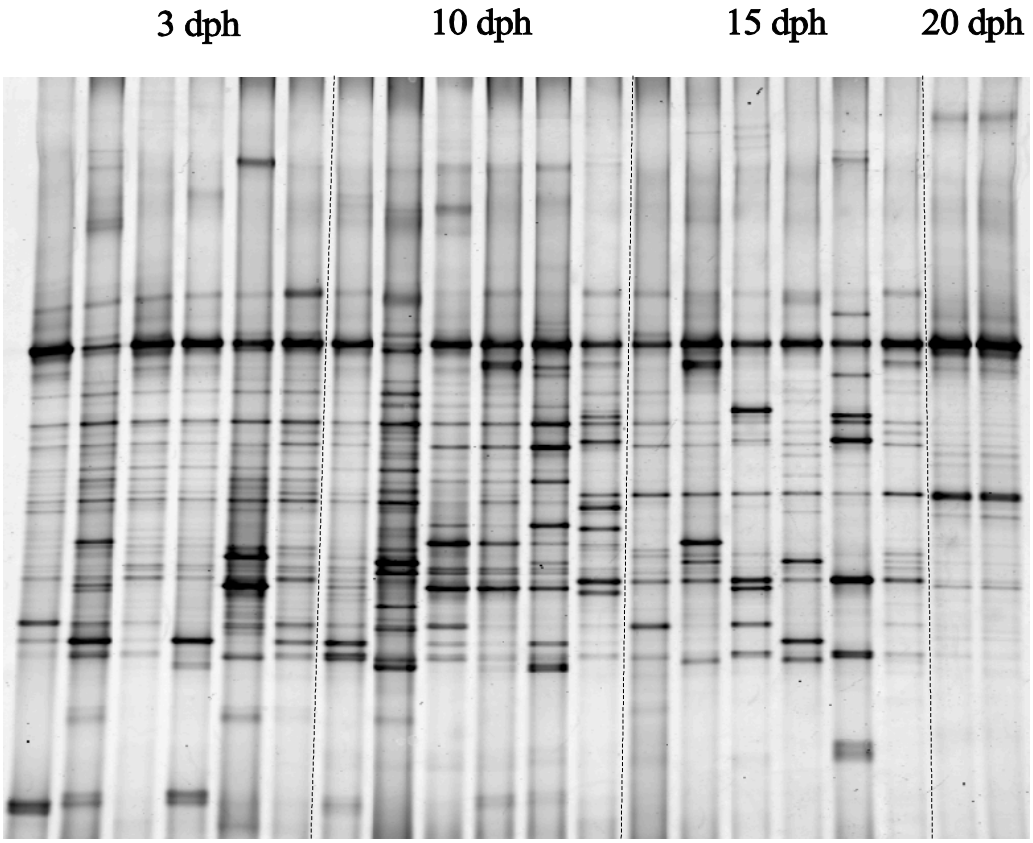

Figure 3 : DGGE profiles illustrating the succession of the bacterial community associated with individual tuna larvae from 3 to 20 dph in Mazarrón in 2010. Each lane corresponded to one larva (Gel VI).

Tables

Table 1
Conditions for the rearing of larvae at the three hatcheries. The information is limited to the periods corresponding to the samples analysed from each hatchery (Years '09 and '10 in brackets)

Hatchery	Palavas (clear water)	Mazarrón (pseudo green)	Heraklion (pseudo green)	Heraklion (mesocosm)
Tank (m^3)	0.5	1.5	0.5	40
Water origin	Sea	Sea	Borehole	Sea + borehole
Daily renewal rate	Closed circuit	50%	Closed circuit with 3% renewal	1% till 8 dph, 3% afterwards
Water inlet rate (h^{-1})	25-30%	15-20%	20-25%	0.042% till 8 dph, 0.125% afterwards
Temperature (°C)	23.5-24 ('09); 24.4 ± 0.5 ('10)	23-26.7	24.5 ± 1	24.0 ± 1
Salinity (psu)	36.1 ± 1.1 ('09); 36.6 ± 1.0 ('10)	37	37	37-40
O_2 concentration (mg l^{-1})	8.1 ± 0.7	5.3-7.2	7.2 ± 0.2	7.2 ± 0.3
pH	7.9 ± 0.4	7.9-8.2	8.2 ± 0.1	7.9 ± 0.2
Water inlet	Bottom	Bottom	Bottom + Lateral, 'V-shaped'	Lateral
Photoperiod (h Light: h Dark)	24L:0D	16L:8D	24L:0D	24L:0D
Illuminance (lux)	300-650	300	300-600	200-5000
Phytoplankton	None	*Nannochloropsis gaditana*	*Chlorella minutissima*	*Chlorella minutissima*
Initial concentration of larvae l^{-1}	85 ('09); 48 ('10)	8	30	1
Initial number of larvae ($\times 10^3$ $tank^{-1}$)	42.5 ('09); 24 ('10)	12	15	40
Rotifer feeding period	3-11 dph	3-17 dph	3 dph onwards	3 dph onwards
Rotifer enrichment	Ori-Green	Ori-Green	Selco® S.pirit	Selco® S.pirit
Artemia feeding period	8 dph onwards	15-19 dph	8 dph onwards	7 dph onwards
Artemia enrichment	Ori-Green (13 dph onwards; '10)	Ori-Green	Selco® S.presso	Selco® S.presso
Yolk sac sea bream larvae as prey	-	18 dph onwards	-	-
Samples provided until	7 dph ('09); 15 dph('10)	12 dph ('09); 20 dph('10)	10 dph ('10)	10 dph ('10)

Table 2
Mean length (mm) and total number of the individuals whose bacterial community was analysed and used in comparisons, as a function of days post hatching (dph) and year of experiment for the three hatcheries,

	Mean length (mm)	Individuals		
		Mazzarón	Heraklion	Palavas
2009				
3 dph	4	6	-	28
7 dph	5	-	-	14
12 dph	7	6	-	-
2010				
Mouth opening	4	10 (3 dph)	9 (3 dph)	15 (2 dph)
10 dph	6	10	20*	22
15 dph	8	7	-	25
20 dph	11	2	-	-

*10 from each condition (Pseudo green or Mesocosm)

Table 3
Median Bray-Curtis similarity within groups based on data from the 14 DGGE gels (the numbers of individuals per group are in brackets), and results from statistical analysis by NPMANOVA for each gel (F values, and *P* levels indicated with asterisks; n.s.: not significant).

Gel	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
2009														
Palavas														
3 dph[§]	0.64 (7)	0.71 (7)	-	-	-	-	-	-	-	-	-	-	0.76 (6)	0.73 (6)
3 dph[@]	0.62 (7)	-	-	-	0.51 (6)	-	-	-	-	-	-	-	0.42 (6)	
7 dph[§]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.39 (14)
Mazarrón														
3 dph	0.80 (6)	0.80 (6)	-	-	-	-	-	-	0.87 (6)	-	-	-	-	-
12 dph	0.66 (6)	-	-	-	0.70 (6)	-	0.65 (6)	-	-	-	-	-	-	-
2010														
Palavas														
2 dph	-	-	0.65 (9)	-	-	-	-	0.71 (8)	-	-	-	-	0.72 (7)	-
10 dph	-	-	-	-	-	-	-	0.54 (8)	-	-	0.56 (8)	-	0.41 (6)	-
15 dph	-	-	-	-	-	-	-	0.84 (9)	-	0.48 (16)	-	-	-	-
Mazarrón														
3 dph	-	-	0.48 (8)	-	-	0.48 (6)	-	-	0.32 (14)	-	-	-	-	-
10 dph	-	-	-	-	-	0.43 (6)	0.73 (8)	-	-	-	0.33 (8)	-	-	-
15 dph	-	-	-	-	-	0.48 (6)	0.33 (6)	-	-	0.45 (7)	-	-	-	-
20 dph	-	-	-	-	-	0.86 (2)	-	-	-	-	-	-	-	-
Heraklion														
3 dph	-	0.63 (7)	0.60 (8)	0.68 (8)	-	-	-	-	-	-	-	-		
10 dph Mesocosm	-	-	-	0.47 (6)	0.63 (4)	-	-	-	-	-	0.32 (4)	0.48 (10)	-	-
10 dph Pseudo green	-	-	-	0.33 (6)	0.59 (4)	-	-	-	-	-	0.27 (5)	0.35 (10)	-	-
NPMANOVA	5.4***	15.9***	5.1***	3.9***	8.2***	2.3***	7.2***	10.6***	8.6***	6.0***	4.9***	1.6 n.s.	5.4***	10.2***

[§] First batch of larvae; [@] second batch

Table 4

Comparison of microbial community profiles between samples from the three hatcheries. Statistical analysis by NPMANOVA based on the Bray-Curtis similarity index (pairwise Bonferroni corrected *P* values, and *P* levels indicated with asterisks).

Stage	2-3 dph	10 dph	15 dph
2009	-	-	-
Palavas/Mazarrón (Gel I)	0.02-0.04*	-	-
Palavas/Mazarrón (Gel II)	0.002**	-	-
2010			
Palavas/Mazarrón (Gel III)	0.0006***	-	-
Palavas/Heraklion (Gel III)	<0.0001***	-	-
Mazarrón /Heraklion (Gel III)	0.003**	-	-
Palavas/Mazarrón (Gel XI)	-	0.0006***	-
Palavas/Heraklion (Gel XI[§])	-	0.002**	-
Mazarrón /Heraklion (Gel XI[§])	-	0.02*	-
Palavas/Mazarrón (Gel X)	-		0.0006***

[§] Other Bonferroni corrected *P* values: 'Palavas' vs. 'Heraklion, pseudo green': 0.005**; 'Palavas' vs. 'Heraklion, mesocosm': 0.01**; 'Mazarrón' vs. 'Heraklion, pseudo green': 0.04*; 'Mazarrón' vs. 'Heraklion, mesocosm': 0.07 n.s.

Table 5

Comparison of microbial community profiles between samples representing different developing stages of tuna larvae. Statistical analysis by NPMANOVA based the Bray-Curtis similarity index (pairwise Bonferroni corrected *P* values, and *P* levels are indicated with asterisks).

Hatchery	Palavas	Mazarrón	Heraklion
2009			
3 dph/7 dph (Gel XIV)	0.0002****	-	-
3 dph/12 dph (Gel I)	-	0.06 n.s.	-
2010			
2 dph/10 dph (Gel VIII)	0.0006***	-	-
2 dph/10 dph (Gel XIII)	0.003**	-	-
2 dph/15 dph (Gel VIII)	0.005**	-	-
10 dph/15 dph (Gel VIII)	0.008**	-	-
3 dph/20 dph (Gel VI)	-	0.2 n.s.[§]	-
10 dph/15 dph (Gel VII)	-	0.002**	-
3 dph/10 dph (pseudo green, Gel IV)	-	-	0.0009***
3 dph/10 dph (mesocosm, Gel IV)	-	-	0.001**

[§] Not significant; the differences between intermediary stages were not significant either

Table 6

Comparison of microbial community profiles in samples from the two consecutive years in Palavas and Mazarrón. Statistical analysis by NPMANOVA based the Bray-Curtis similarity index (pairwise Bonferroni corrected *P* values, and *P* levels are indicated with asterisks).

Hatchery	Palavas	Mazarrón
'09(3dph)[§]/'10 (2 dph, Gel XIII)	0.002-0.004**	-
'09/'10 (3 dph, Gel IX)	-	<0.0001***
'09(12 dph)/'10 (10 dph, Gel VII)	-	0.0006***
'09(12 dph)/'10 (15 dph, Gel VII)	-	0.007**

[§] Significant dissimilarity between the two batches in '09 (*P* = 0.01*)

Table 7

Spatio-temporal variations of the indices of microbial diversity in individual larva. Data are given as a function of developmental stage for the two years of investigation in the three hatcheries (‘09 or ’10; means ± standard errors). The mean band richness is based on the presence of bands detected per individual, whereas the other indices of diversity were computed from relative peak intensities. Tests for significant differences between two means were done with t-test or Mann-Whitney test (MW), or in case of multiple comparisons with ANOVA followed by Tukey multiple comparison test, or with Kruskal-Wallis test (KW) followed by Dunn test. The type of test used depended on the normality and the equality of variance assumptions for the parametric tests. *P* levels are indicated with asterisks (n.s.: not significant). For each comparison the means without common superscript are significantly different.

Table 7

Stage	Group	Replicates	Dominance	Band richness	Shannon	Evenness
2-3 dph						
	Palavas '09§	14	$0.26^{a} \pm 0.02$	$16.8^{ab} \pm 0.7$	$2.05^{b} \pm 0.07$	$0.49^{c} \pm 0.03$
	Palavas '09@	8	$0.28^{a} \pm 0.05$	$14.7^{b} \pm 1.4$	$2.01^{b} \pm 0.18$	$0.58^{bc} \pm 0.03$
	Palavas '10	9	$0.15^{b} \pm 0.01$	$19.5^{a} \pm 0.8$	$2.61^{a} \pm 0.04$	$0.72^{a} \pm 0.02$
	Mazarrón '09	6	$0.23^{ab} \pm 0.01$	$13.5^{b} \pm 0.6$	$2.05^{b} \pm 0.03$	$0.59^{bc} \pm 0.01$
	Mazarrón '10	20	$0.18^{b} \pm 0.01$	$17.7^{ab} \pm 0.9$	$2.43^{a} \pm 0.07$	$0.67^{ab} \pm 0.03$
	Heraklion '10	8	$0.22^{ab} \pm 0.01$	$16.1^{ab} \pm 0.8$	$2.29^{ab} \pm 0.08$	$0.66^{ab} \pm 0.02$
			KW***	KW**	ANOVA***	ANOVA***
7 dph	Palavas '09§	14	0.27 ± 0.03	15.8 ± 1.2	1.93 ± 0.11	0.46 ± 0.03
10 dph						
	Palavas '10	10	$0.15^{b} \pm 0.02$	$20.0^{a} \pm 0.5$	$2.59^{a} \pm 0.06$	$0.69^{a} \pm 0.02$
	Mazarrón '10	15	$0.17^{ab} \pm 0.01$	$20.2^{a} \pm 1.2$	$2.59^{a} \pm 0.10$	$0.70^{a} \pm 0.04$
	Heraklion '10	18	$0.23^{a} \pm 0.03$	$13.0^{b} \pm 0.6$	$2.09^{b} \pm 0.09$	$0.66^{a} \pm 0.02$
			KW*	KW***	KW***	ANOVA (n.s.)
12 dph	Mazarrón '09	6	0.29 ± 0.04	16.4 ± 1.0	2.07 ± 0.14	0.54 ± 0.04
15 dph						
	Palavas '10	16	$0.13^{b} \pm 0.01$	$19.9^{a} \pm 0.9$	$2.75^{a} \pm 0.05$	$0.82^{a} \pm 0.03$
	Mazarrón '10	7	$0.16^{a} \pm 0.02$	$15.3^{b} \pm 0.4$	$2.39^{b} \pm 0.06$	$0.73^{a} \pm 0.04$
			MW*	MW**	MW***	t (n.s.)
20 dph	Mazarrón '10	2	0.27-0.30	11-13	1.74-1.88	0.52-0.55

§ First batch of larvae; @ second batch