Heterotrophic Bacterial Flora Associated with European Eel *Anguilla anguilla* Reared in Freshwater

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The heterotrophic bacterial microflora of European eel reared in freshwater and its surrounding aqueous environment is reported. Quality of eel culture water is also discussed with respect to its physicochemical and bacteriological characteristics. The presence of bacterial species pathogenic for eel and its serological classification was investigated in fishery water and eels. Overall 675 isolates from fishery water and healthy or diseased eel were identified at genus level. *Pseudomonas* was the most frequently encountered genus. *Moraxella* sp. and *Acinetobacter* sp. were abundant in fishery water, and *Aeromonas* sp. in elver and internal organs of silver eel. *Plesiomonas* sp., *Escherichia* sp., *Enterobacter* sp., *Serratia* sp., *Citrobacter* sp., and *Streptococcus* sp. were minority genera that were only recovered from eel. The species *Aeromonas hydrophila*, *A. sobria*, and *Alteromonas putrefaciens* were mostly isolated from diseased eel whereas other potential fish pathogens such as *P. fluorescens*, *V. anguillarum*, and *Plesiomonas shigelloides* were generally observed in fishery water or healthy eel. Following the typing scheme of Guinée and Jensen, most of the isolates of *A. hydrophila* from diseased European eels, as well as the strain *A. hydrophila* Y-62 pathogenic for Japanese eel, belonged to O19 serogroup. A relationship between origin and serogroup for *A. sobria* was not observed. None *Vibrio anguillarum* isolates agglutinated with O1 and O2 antisera of Sorensen and Larsen.

Characterization of the bacterial communities related to eel rearing systems can be useful to understand the onset of infectious diseases, since this microflora may include opportunistic pathogens with an active role in the development of epizootics. On the other hand, the number of epizootic outbreaks produced by obligate pathogens of eel such as *Pseudomonas anguilliseptica* and *Vibrio vulnificus* biotype 2 so far is limited.¹⁻⁴⁾

Aeromonas hydrophila is one of the most important species potentially pathogenic for fish. This bacterium is frequently isolated from aquatic ecosystems, and it is considered to have worldwide distribution. Many reports have recognized it as pathogen from a wide variety of freshwater fish species. Among them Kou⁵⁾ in Taiwan, Rickards⁶⁾ in Japan, Levanon *et al.*⁷⁾ in Israel, and Møllergaard and Dalsgaard⁸⁾ in Denmark reported on *Aeromonas hydrophila* as a causative agent of epizootic outbreaks in eels. Recent studies suggest that motile *Aeromonas* strains can be separated serologically and antigenically in various groups.^{9,10)} These seroepidemiolo-

gical results could be crucial for planning effective vaccination programmes. Nevertheless, descriptions of the normal bacterial flora of fish and its artificial rearing environment are almost limited to those carried out with salmonids.^{11,12)}

We have carried out a study of the heterotrophic bacterial flora of eel culture water, elvers, and silver eels at an eel breeding plant near Valencia (Spain) over a one year period. We have also studied the bacterial species recovered from epizootic outbreaks and their serological classification. The physicochemical parameters of the eel culture water were investigated in order to evaluate its quality to rear eels.

Material and Methods

European Eel Farm

The eel farm is located in Valencia, on the Mediterranean coast of Spain. European eels were stocked at 30 kg/m³ in concrete tanks of 1.30 to 1.80 m diameter and 0.70 m depth supplied with spring freshwater without any system to recirculate or heat the inflowing water.

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Sample Collection

Samples of eel culture water (inflow and tank) and eels (elvers and silver eels) were taken at the mentioned eel farm from April 1987 until March 1988. Inflowing and tank water were collected 5 cm below the surface in sterile 500 ml glass bottles. Fifty elvers (average weight 0.3 g) were chosen from a random sample from each tank studied. These were washed by vigorous agitation in sterile saline solution (SS) (0.9% w/v NaCl) to remove the surface microflora, and the elvers were then homogenized in sterile SS. Samples of elver homogenate were used for bacteriological analysis. Three silver eels (weight 7-72 g) were randomly chosen from each tank studied. Samples of kidney, liver, and gills from each silver eel were used for bacteriological analysis.

Physico-chemical Parameters of Water

Salinity was measured *in situ* using a salinometer YSI model 33SCT. Dissolved oxygen and water temperature were determined *in situ* using dissolved oxygen+temperature meter (Syland). Hydrogen ion activity was measured *in situ* using a pH meter Orion SA250. Biological oxygen demand (BOD₅) was determined in the laboratory with an Orion 97-08 oxygen electrode.¹³⁾ Determinations of nitrate and nitrite ions were performed by Griess's method, ammonium ion was measured with an Orion 95-10 ammonium electrode.¹³⁾

Bacteriological Analysis

1. Faecal indicator microorganisms: CFU (colony forming unit) of faecal coliforms and enterococci in samples of inflowing and tank

waters were determined by membrane filtration culture method.¹³⁾

2. Viable cell counts and isolation of bacterial strains on non selective media: CFU of heterotrophic mesophilic bacteria in samples of inflowing and tank waters, and elvers homogenate were determined by plate count method¹³⁾ on tryptone soya agar (TSA) (Oxoid), with incubation at 22°C for seven days. Isolation of bacterial strains was made on TSA plates with >30 to 300 colonies. 46% of colonies from inflowing water, 56% of colonies from tank water, and 71% of colonies from elver homogenate were picked off and subcultured repeatedly on fresh media until purification was achieved. Samples of gills, liver, and kidney from silver eel were streaked directly on duplicate plates of TSA and brain heart infusion (BHI) (Difco) agar, and incubated as described previously. Three colonies of each morphological type were picked off and purified on TSA plates.

3. Isolation of bacterial strains on selective media: Samples of inflowing and tank waters, elvers homogenate, as well as gills, liver, and kidney from silver eels were streaked on duplicate plates of MacConkey agar (Oxoid) and thiosulphate citrate bile sucrose (TCBS) agar (Oxoid). The plates were incubated at 28°C for two days and then three colonies of each morphological type were picked off and streaked in TSA plates.

Bacterial Identification

Bacterial isolates were identified to genus level using Bergey's Manual of Systematic Bacteriology (Vols. 1 and 2). The routine identification included the following tests: colony pigmentation,

Table 1. Physicochemical parameters of inflowing and tank waters

Date/Fish holding tank	Water sample	Temperature (°C)	Dissolved oxygen (ppm)	pH	Salinity (%)	BOD ₅ (mg/l O ₂)	NO ₃ ⁻ (ppm)	NO ₂ ⁻ (ppm) × 10 ³	NH ₄ ⁺ (ppm) × 10 ³
April, 87									
T1	Inflow	19.0	7.1	7.3	0.1	ND*	74.8	3	18
	Tank	19.0	6.7	7.2	0.5	ND	80.2	208	18
August, 87									
T4	Inflow	19.7	5.3	7.1	0.1	1.7	50.0	30	81
	Tank	20.9	8.6	7.4	0.5	3.2	45.0	180	3
December, 87									
T1	Inflow	17.0	ND	7.5	0.1	1.7	45.9	28	2
	Tank	17.0	ND	7.5	0.1	1.7	45.9	9	162
February, 88									
T1	Inflow	17.0	6.7	7.4	0.1	1.0	29.1	9	19
	Tank	16.6	5.1	7.2	0.1	2.0	31.0	93	178

* Not determined.

motility, gram reaction, oxidase, O/F metabolism, catalase, nitrate reduction, gelatin liquefaction, indole production, and SH_2 production. API system 20E was used for identification of enterobacteria to the species level. Additional tests used to identify *Vibrio*, *Plesiomonas*, and *Aeromonas* species were based on the schemes of Poppof¹⁴⁾ and Lee & Donovan.¹⁵⁾ O/129 sensitivity (150 μg), gas production from glucose, Moeller's decarboxylases for arginine, lysine, and ornithine, growth in 0% and 6% NaCl (w/v), acid production from arabinose, salicin, and arbutin, Voges-Proskauer, and hydrolysis of esculin. Fluorescent *Pseudomonas* species were identified using the tests reported by Palleroni.¹⁶⁾ Pyoverdine production, Thornley's decarboxylase for arginine, nitrite reduction, DNase production, and growth at 42°C.

Serology

Slide agglutination method was used. Twenty-two isolates of *P. fluorescens* (seventeen from eel and five from tank water) and seven isolates of *Pseudomonas* sp. from fish were serologically tested with the antiserum made against the Japanese strain of *P. fluorescens* O3L (Dr. T. P. Nieto, Spain). Fifteen isolates of *A. hydrophila*

(thirteen from eel and two from tank water), nineteen isolates of *A. sobria* (thirteen from eel and six from water), and six isolates of *Aeromonas* sp. from tank water were serologically tested with thirty O antisera¹⁰⁾ (Dr. A. M. Guinée, The Netherlands). All *Vibrio anguillarum* isolates were serologically tested with O1 and O2 antisera,¹⁷⁾ (Dr. J. L. Larsen, Denmark). *Pseudomonas fluorescens* O3L (*Tilapia nilotica*, Dr. H. Motoki, Japan), *Vibrio anguillarum* NCMB 6^T and RV22 (*Scophthalmus maximus*, Dr. M. L. Lemos, Spain), and *Aeromonas hydrophila* Y-62 (*Anguilla japonica*, Dr. T. Aoki, Japan) were used as reference.

Results

Quality of Eel Culture Water

Temperature of eel culture water ranged from 17°C during autumn and winter, to 21°C in summer. The pH values of inflowing and tank water ranged from 7.1 to 7.5. Biological oxygen demand ranged from 1.04 to 1.70 mg/l O_2 for inflowing water, and 1.73-3.15 mg/l O_2 for tank water. Levels of nitrate in eel culture water fluctuated between 29.1 and 80.2 mg/l. Levels of nitrite ranged from 10^{-3} to 10^{-1} mg NO_2^- .

Table 2. Bacterial counts in water and elver samples*1

Date/Fish holding tank	Sample	Heterotrophic bacteria	Faecal coliforms	Faecal streptococci
April, 87				
	Inflowing water	2.6×10^5	0	0
T1	Tank water	1.2×10^4	0	0
T1	Elver	3.0×10^5	ND*2	ND
August, 87				
	Inflowing water	2.6×10^5	0	1.2×10^1
T4	Tank water	1.2×10^4	0	$\geq 1.2 \times 10^1$
December, 87				
	Inflowing water	1.0×10^5	0	2
T1	Tank water	2.1×10^4	2.3×10^1	8.7×10^5
T1	Elver	3.2×10^5	ND	ND
February, 88				
	Inflowing water	$\geq 1.0 \times 10^5$	0	3
T1	Tank water	2.5×10^5	0	3.2×10^4
T1	Elver	1.2×10^6	ND	ND
March, 88				
T1	Tank water	1.0×10^4	0	0
T2	Tank water	7.2×10^3	0	0
T3	Tank water	6.0×10^3	0	0
T1	Elver	$\geq 1.0 \times 10^6$	ND	ND
T2	Elver	2.0×10^6	ND	ND
T3	Elver	1.0×10^6	ND	ND

*1 Water samples, colony forming units per millilitre; elver samples, colony forming units per gram.

*2 Not determined.

Table 3. Bacteria isolated from water and fish samples on TSA, BHI agar, MacConkey, and TCBS plates throughout the study

Date/ Samples* ¹	Total isolates	<i>Moraxella-Acinetobacter</i>	<i>Pseudomonas</i>	<i>Flavobacterium</i>	<i>Janthinobacter</i>	<i>Alteromonas</i>	<i>Aeromonas</i>	<i>Vibrio</i>	<i>Plesiomonas</i>	<i>Enterobacteriaceae</i>	<i>Staphylococcus</i>	<i>Streptococcus</i>	<i>Micrococcus</i>	<i>Bacillus</i>
April, 87* ²														
IW	14	0	4	2	1	0	1	3	0	0	0	0	2	1
TW	28	2	17	1	0	0	0	0	0	0	1	0	4	3
HE	34	0	3	0	0	0	16	1	0	0	3	0	9	2
DE	32	0	14	0	0	0	9	1	0	0	0	0	7	1
August, 87* ²														
TW	22	0	12	2	0	0	1	1	0	0	1	0	2	3
GHSE	14	1	10	1	0	0	0	0	0	0	1	0	1	0
LHSE	15	0	0	0	0	0	3	0	0	2	3	5	2	0
GDSE	38	0	16	3	0	3	6	2	0	3	2	0	2	1
LDSE	16	0	6	0	0	1	2	0	0	1	3	1	2	0
December, 87														
IW	7	1	2	0	0	0	0	0	0	0	0	0	2	2
TW	63	20	7	9	0	1	8	11	0	2	0	0	3	2
HE	72	2	12	0	0	1	39	5	0	13	0	0	0	0
January, 88* ²														
TW	21	0	0	0	0	0	11	10	0	0	0	0	0	0
LDSE	8	0	1	1	0	6	0	0	0	0	0	0	0	0
February, 88														
IW	10	8	1	0	0	0	0	0	0	0	0	0	0	1
TW	74	8	55	2	0	1	8	0	0	0	0	0	0	0
HE	74	8	47	0	0	2	7	1	1	8	0	0	0	0
GHSE	16	1	9	0	0	0	6	0	0	0	0	0	0	0
LHSE	56	2	41	1	0	1	6	0	2	3	0	0	0	0
March, 88* ²														
TW	27	2	2	0	0	2	6	9	0	3	1	0	1	1
DE	29	0	9	0	0	0	15	0	0	3	1	0	0	1

*¹ Analyzed samples; IW, inflowing water; TW, tank water; HE, healthy eelers; DE, diseased eelers; GHSE, gills of healthy silver eels; LHSE, liver of healthy silver eels; GDSE, gills of diseased silver eels; LDSE, liver of diseased silver eels.

*² Epizootic outbreak.

N/I for inflowing and tank waters (Table 1). The amounts of viable heterotrophic bacteria, faecal coliforms, and enterococci in eel culture water are summarized in Table 2. Viable counts of heterotrophic bacteria of inflowing water (10^2 - 10^3 colony forming unit, cfu/ml) were lower than those of tank water (10^3 - 10^6 cfu/ml). These counts remained relatively constant throughout the sampling period. Faecal coliforms appeared always in low numbers, 23 cfu/ml being the highest value recorded. On the other hand, counts of enterococci were low (10^1 cfu/ml) in inflowing water, but they increased to 10^2 - 10^4 cfu/ml in tank water.

Generic Composition of Heterotrophic Bacteria

The bacterial groups isolated from eel culture water and eels are listed in Tables 3 and 4. *Pseudomonas* was the most encountered genus. Furthermore, *Vibrio* sp., *Micrococcus* sp., *Bacillus* sp., and *Flavobacterium* sp. were usually recovered from all kinds of samples in low numbers (Table 3). Some differences between the bacterial microflora of eel culture water and eels were observed. Inflowing and tank waters showed the highest percentages of isolates of *Moraxella* and *Acinetobacter*, but the lowest ones of *Aeromonas*. In contrast, the highest percentages of isolates of *Aeromonas*, as well as the lowest ones of *Moraxella*

Table 4. Generic distribution of the bacteria recovered from water and fish samples on TSA plates

Bacteria	Inflowing water	Tank water	Elver	Silver eel
<i>Moraxella</i>	0*	12 (8.2)	1 (0.5)	1 (0.8)
<i>Acinetobacter</i>	8 (36.4)	18 (12.2)	10 (4.8)	1 (0.8)
<i>Pseudomonas</i>	5 (22.7)	78 (53.0)	77 (37.2)	66 (54.1)
<i>Flavobacterium</i>	1 (4.5)	14 (9.5)	0	5 (4.1)
<i>Janthinobacter</i>	1 (4.5)	0	0	0
<i>Alteromonas</i>	0	1 (0.7)	3 (1.4)	4 (3.3)
<i>Aeromonas</i>	1 (4.5)	6 (4.1)	67 (32.4)	14 (11.5)
<i>Vibrio</i>	3 (13.6)	2 (1.4)	8 (3.9)	2 (1.6)
<i>Plesiomonas</i>	0	0	1 (0.5)	1 (0.8)
<i>Escherichia</i>	0	0	5 (2.4)	3 (2.5)
<i>Enterobacter</i>	0	0	8 (3.9)	2 (1.6)
<i>Serratia</i>	0	0	2 (1.0)	0
<i>Citrobacter</i>	0	0	3 (1.4)	0
<i>Staphylococcus</i>	0	2 (1.4)	3 (1.4)	9 (7.4)
<i>Streptococcus</i>	0	0	0	6 (5.0)
<i>Micrococcus</i>	2 (9.1)	9 (6.1)	17 (8.3)	7 (5.7)
<i>Bacillus</i>	1 (4.5)	6 (4.1)	3 (1.4)	1 (0.8)

* Number of isolates (relative frequencies).

Table 5. Species distribution in eel culture water (inflow and tank) and eel (elver and silver eel)

Species	Eel culture water	Healthy eel	Diseased eel
<i>P. fluorescens</i>	14 (13.9)*	79 (78.2)	8 (7.9)
<i>P. putida</i>	31 (56.4)	24 (43.6)	0
<i>A. putrefaciens</i>	4 (25.0)	2 (12.5)	10 (62.5)
<i>A. hydrophila</i>	5 (21.7)	7 (30.4)	11 (47.8)
<i>A. sobria</i>	5 (19.2)	6 (23.1)	15 (57.6)
<i>A. caviae</i>	11 (24.4)	33 (73.3)	1 (2.2)
<i>V. anguillarum</i>	7 (100)	0	0
<i>P. shigelloides</i>	0	3 (100)	0

* Number of isolates (relative frequencies).

and *Acinetobacter* were observed in elvers and silver eels (Table 4). Other genera such as *Escherichia*, *Serratia*, *Citrobacter*, *Plesiomonas*, and *Streptococcus* were only isolated from elvers and internal organs of silver eels (Tables 3 and 4).

Bacterial Species Recovered from Diseased European Eels and Its Serotyping

The population of elvers and silver eels suffered four epizootic outbreaks throughout the sampling period. In general, the outbreaks lasted 1–2 weeks, and the mortality reached nearly 80% of the eels stocked in each tank. Pathological signs such as petechial haemorrhages on the gill region and ventral side of the body, reddening of the fins, pale and haemorrhagic liver, and swollen kidney were observed in moribund eels. On January, pure cultures of *Alteromonas putrefaciens* were recovered from kidney and liver of silver eels during an outbreak of red fin disease. The diseased eels had been treated by the farmer

with oxytetracycline, tetracycline, and trimethoprim/sulphonamides before the sampling was made. Mixed cultures were usually isolated from diseased eels during the rest of epizootics (Table 3). However, the species *A. hydrophila* and *A. sobria*, as well as *Alteromonas putrefaciens* were encountered preponderant in diseased eel samples (Table 5). Other potential fish pathogens such as *P. fluorescens*, *Vibrio anguillarum*, and *Plesiomonas shigelloides* were mainly recovered from healthy eel or eel culture water (Table 5). Furthermore, the species *P. putida* and *A. caviae* were always recovered from the studied samples in absence of epizootics (Table 5).

None of the *P. fluorescens* isolates agglutinated with the antiserum made against the pathogenic reference strain O3L. Interestingly, most of the typable isolates of *A. hydrophila* from diseased eels (five out of eight isolates), as well as the reference strain Y-62 pathogenic for Japanese eels, belonged to the O19 serogroup of Guinea

and Jansen.¹⁰⁾ In contrast, isolates of *A. hydrophila* O6 and O9 were associated to healthy eels. The serotype O24 was mostly encountered among the typable isolates of *A. sobria* from all kinds of samples (seven out of sixteen isolates). Some *A. sobria* isolates from healthy and diseased eels belonged to the serotypes O4, O6, O11, O15, O16, and O29 of Guinée and Jansen.¹⁰⁾ Our *V. anguillarum* isolates did not agglutinate with antisera O1 and O2 of Sorensen and Larsen.¹⁷⁾

Discussion

The quality of eel culture water was found suitable for rearing eels with regard to its physico-chemical parameters, which fell within those recorded by Gault.¹⁸⁾ However, temperature of tank water was always lower than 22-26°C, optimal for the growth of *Anguilla anguilla*.¹⁸⁾ The values of biological oxygen demand in tank water were always higher than those in inflowing water, as a consequence of the organic contamination of the water caused by the intensive rearing. The amounts of viable heterotrophic bacteria in eel culture water (inflow and tank) maintained themselves within the limits described for unpolluted freshwater.¹⁹⁾ As has been previously reported by Austin and Allen-Austin,²⁰⁾ we have observed no variations of the bacterial population size related to water temperature increases. Faecal bacteria in eel culture water seemed to be introduced by diet since faecal coliform counts were always low and enterococci counts fluctuated from 10¹ to 10⁴ cfu/ml. The importance of food-borne enterococci contamination of water, as well as the incidence of streptococciosis outbreaks in Japanese eels has been highlighted by Taniguchi.²¹⁾

Although the genus *Pseudomonas* was preponderant in all samples studied, we observed differences between the eels and the eel culture water with regard to their generic composition. Eel microflora was characterized by the high percentage of bacteria related to an intestinal habitat, such as *Aeromonas*, *Plesiomonas*, and enterobacteria which have been reported as saprophytic flora of the gastrointestinal tract of salmonids and warm-water fishes.^{12, 22, 23)} Eel culture water microflora was characterized by the low levels of bacterial genera of intestinal origin and also by the abundance of bacteria belonging to *Moraxella* and *Acinetobacter*.

Opportunistic fish pathogens were isolated not only in the presence of epizootic outbreaks, but

also in their absence. However, the species *A. hydrophila*, *A. sobria*, and *A. putrefaciens* were mainly associated with diseased eels. *Alteromonas putrefaciens* isolates were related to an infectious disease in silver eels treated with oxytetracycline, tetracycline, and trimethoprim/sulphonamides in the farm. Until now this species has been associated with spoilage of fish fillets,²⁴⁾ but there are very few reports on its role as a member of the bacterial microflora of healthy and diseased living fishes.²⁵⁾ Nevertheless when the virulence was evaluated by experimental infection of fingerlings (5-8 g), the isolates of *A. hydrophila* were revealed to be the most pathogenic ones for European eel, followed by those of *A. sobria*, whereas *A. putrefaciens* strains were avirulent for elvers (unpublished data). Isolates of other species described as fish pathogens such as *Vibrio anguillarum*²⁶⁾ and *Plesiomonas shigelloides*²⁷⁾ were always recovered from eel culture water or healthy eels. Furthermore, we observed that the species *P. fluorescens*, *P. putida*, and *A. caviae* were mainly recovered from the studied samples in the absence of epizootics.

Following the typing scheme of Guinée and Jansen¹⁰⁾ we have observed that serotype O24 was preponderant in *A. sobria* isolates, but no relationship between origin and serogroup was observed for this species. The isolates of *A. hydrophila* O19 were always recovered from eels and tank water during epizootic outbreaks. Interestingly, the Spanish isolates of *A. hydrophila* associated with diseased *Anguilla anguilla*, as well as the Japanese strain Y-62 pathogenic for *Anguilla japonica* belonged to the O19 serogroup.

Further studies are in progress to investigate the relationship between pathogenicity and serotype of these *A. hydrophila* strains, as well as the possible importance of the latter as an epidemiological marker.

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