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in

Zrncic S. (ed.).
Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture

Zaragoza : CIHEAM
Options Méditerranéennes : Série B. Etudes et Recherches; n. 75

2020
pages 83-96

Article available on line / Article disponible en ligne à l'adresse :

http://om.ciheam.org/article.php?IDPDF=00007943

To cite this article / Pour citer cet article

Varvarigos P. **10. Photobacterium damsela subsp. piscicida.** In : Zrncic S. (ed.). *Diagnostic Manual for the main pathogens in European seabass and Gilthead seabream aquaculture.* Zaragoza : CIHEAM, 2020. p. 83-96 (Options Méditerranéennes : Série B. Etudes et Recherches; n. 75)

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10. *Photobacterium damsela* subsp. *piscicida*

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10.1. Aetiology of pasteurellosis (photobacteriosis)

Photobacterium damselae subsp. *piscicida* (former name: *Pasteurella piscicida* (Gauthier *et al.,* 1995; Jansen and Surgalla, 1968) causes a disease of importance resulting in serious losses among cultured fish species in Europe, such as gilthead seabream (*Sparus aurata*), red porgy (*Pagrus pagrus*), red seabream (*Pagrus major*), European seabass (*Dicentrarchus labrax*), meagre (*Argyrosomus regius*) and sole (*Solea* spp.). Gilthead seabream and European seabass are suffering most of the economic losses under aquaculture conditions in Europe including countries such as France, Italy, Malta, Spain, Portugal and Greece (Toranzo *et al.,* 1991; Baudin-Laurencin *et al*., 1991; Ceschia *et al.,* 1991; Baptista *et al*., 1996; Bakopoulos *et al.,* 1997; Zorilla *et al*., 1999).

The disease is also of importance in Turkey (Candan *et al*., 1996; Korun and Timur, 2005) as

well as Japan (Kusuda and Yamaoka, 1972; Koike *et al.,* 1975) and the USA, where it was first isolated from white perch (*Roccus americanus*) by Janssen and Surgalla, 1968.

Photobacterium damselae subspecies *damsela* (*Ph.d.d.*) *and Photobacterium damselae* subspecies *piscicida* (*Ph.d.p*) belong to the thermotolerant group of the genus *Photobacterium* and are genotypically homogeneous subspecies of *Photobacterium damselae*, based on small-subunit rRNA sequencing and DNA:DNA hybridization (Romalde, 2002). The pathogenic subspecies *piscicida* (*Ph.d.p.*) is phenotypically and serologically homogeneous and is the only non-flagellated member of the genus.

Amplified Fragment Length Polymorphism (AFLP) can help epizootiological and taxonomic studies of the highly homogeneous subspecies *Photobacterium damselae* subspecies *piscicida* (Kvitt *et al*., 2002).The combination of PCR direct amplification of a 16S rRNA gene sequence and AFLP in a 2-step procedure grouped *Ph.d.p.* isolates from different geographic regions into distinct clusters on the basis of AFLP intraspecific polymorphisms. The Japanese isolates of *Ph.d.p*. were distinguished from the Mediterranean/European isolates from Italy, Spain, Greece and Israel at a cut-off value of 83% similarity. Further subclustering of the Med/European isolates at a cut-off value of 97% discriminated the Italian, Spanish and Greek isolates from the Israeli isolates.

10.2. Clinical diagnosis

Pasteurellosis presents itself in the hatcheries as hyperacute or acute septicaemia. Seabream larvae, juveniles or fry are often found dead in large numbers on the bottom of the tank with only a few darker fish swimming sluggishly and off-balance near the surface, often showing nervous convulsions (bacterial encephalopathy) prior to death (Abu-Elala *et al*., 2015).

During acute cases of photobacteriosis, fish exhibit only a few pathological signs. Usually, there are no alarming signs with fish behaving and feeding normally before the disease strikes; hence, many among the dead fish carry amounts of feed in their stomach and gut. Convulsive erratic swimming prior to death often comprise the only clinical signs of the acute outbreaks, where internal lesions are often absent at necropsy. Anorexia, lethargy, darkening and ulceration of the skin follow shortly afterwards.

As the disease progresses the gills become pale with excessive mucous secretions with congested inflamed patches and often focal necroses next to congested areas. Lip, opercula skin and lower jaw inflammation and necrotic skin patches on the body flanks, dorsal area and tail become common. The fins, mainly pelvic, dorsal and caudal may be eroded. Skin and fin erosions are covered with mucous, thus the lesions appear in the water as white patches. Overall, there is no haemorrhagic appearance. The liver is most often inflamed and congested; the spleen is enlarged (splenomegaly) and the kidney pale and oedematous. The intestine carries a moderate quantity of fluid and some whitish mucous clots. The swim bladder is not distended, thus, the majority of dead fish have sunk to the bottom of the tank or cage.

In the more chronic form of the disease typical pseudotuberculi develop mainly in the spleen and/or kidney parenchyma. They comprise creamy-white granulomatous nodules, composed of masses of bacterial cells, epithelial cells, fibroblasts, phagocytes and necrotic cell debris. These have led to the descriptive name "pseudotuberculosis". Bacteria accumulate in phagocytes, capillaries and interstitial spaces (Andreoni and Magnani, 2014) and bacteraemia is pronounced.The gill, skin and fin epithelial lesions are suggestive of their susceptibility to bacterial exotoxins as the bacteria gain entrance into the fish body during horizontal transmission.

10.3. Epizootiology of pasteurellosis (photobacteriosis)

The bacteria spread via infected phagocytes, mainly macrophages and the spread is rapid with lethal effects after a few days of infection (Barnes and Ellis, 2004). The disease is hard to eradicate by antibiotic treatments due to the intracellular location of the bacterium, but also due to transferable genetic elements (R plasmids) carrying genes of resistance to many antibiotics (Andreoni and Magnani, 2014). The intracellular location seems to protect from circulating antibodies after vaccination against the disease and may explain the low RPS conferred by vaccination as well as the short duration of immunity post-vaccination. Carriers persist on farms and may show disease under stressful conditions (personal observations). In addition, surviving fish from natural or experimental infection are not protected during subsequent re-challenge (Barnes and Ellis, 2004). Iron acquisition from its host, by means of iron-binding siderophores, increase virulence and the cytotoxic extracellular products (ECPs) damage the infected cells with the consequent release of the bacterium and the invasion of adjacent cells (Andreoni and Magnani, 2014).

A variety of marine fish are natural hosts of the pathogen (Romalde, 2002), but the exact behaviour of the organism outside the host is unknown. *Ph.d.p.* strains are able to survive in culturable state in sea water and sediment for only 6 to 12 days (Magarinos *et al.*, 1994), but virulent *Ph.d.p.* cells can enter a "viable but not culturable" (VBNC) state in response to environmental stresses, such as starvation, antibiotic exposure, or low temperature, that allows the cell to enter a state of dormancy and survive until conditions allow resuscitation and reinitiation of infection (Magarinos *et al.*, 1994; Oliver, 2010; Pinto *et al.*, 2015).

The ability to enter a VBNC state is also common for the survival of virulent strains of the subspecies *damsela*. *Ph.d.d.* maintains infectivity in sea water and sediments for at least 1 year (Fouz *et al.*, 1998). Hence, water and sediments can also act as reservoirs for virulent *Ph.d.d.* strains.

Pasteurellosis, or rather photobacteriosis, is a temperature-dependent septicaemic disease. Outbreaks occur when the water temperature rises above 18^{o}C (Korun and Timur, 2005). Below this temperature fish may be subclinical carriers harbouring the pathogen for long periods (Romalde, 2002). In grow-out facilities, pasteurellosis outbreaks occur from late spring or early summer until late autumn, while sea water temperature is maintained above 20°C. In hatcheries, where warm borehole water (>18°C) is in use, photobacteriosis is a major threat all year round.

There is no fish species-specific characteristic lesion other than the differences in sensitivity of age classes among species (Andreoni and Magnani, 2014).

Seabream is susceptible when very young or around the weaning stage of juveniles and remains very sensitive until the size of 6g. Its sensitivity gradually decreases from then onwards, thus, for this species, photobacteriosis is mostly a problem in the hatchery/nursery and during the first months in the grow-out facilities, especially when the transfer to cages coincides with the warm season.

Seabass is susceptible to pasteurellosis beyond the size of 1g (nursery stage onwards). The disease causes the highest mortalities in caged seabass between 5g and 40g. Thus, for bass, photobacteriosis is mostly a problem during the first summer and autumn in the grow-out facilities. Nevertheless, despite the gradual lowering of losses as the seabass grow, they remain considerably susceptible until harvest.

10.4. Sampling

10.4.1. Preparation and shipment of samples from fish

Marginalized, lethargic, moribund fish showing pronounced external lesions or behavioural abnormalities, but not dead specimens, should be collected and sent refrigerated to the laboratory for delivery within 12-24h (fast courier). It is best to collect the fish prior to administering any antibacterial treatment.

Samples must be shipped according to the procedure described in Chapter 2.2.

Freshly dead fish, target organs (e.g. spleen, liver, kidney), fish eggs, live prey organisms, or even tank and filter sediments may be submitted for PCR examination to confirm the presence of the pathogen. These may be placed in screw-cap tubes of appropriate size. The samples may be fixed in RNA later (1:5 v/v), or simply kept deep-frozen at below -20°C until dispatch. If dispatch is planned on the same day, the samples may simply be kept refrigerated.

When running PCR for diagnostic purposes, the nucleic acid (NA) extraction step prior to its targeted amplification procedure is of utmost importance. The pooling of target tissues most likely to be infected by the pathogen (spleen, kidney, liver, brain), or whole specimens, when very small, must not be excessive and over-dilute the pathogen if assumed present in a small fraction of the pooled tissues.

At the laboratory, meticulous homogenization of the tissues ensuring cell lysis is important and the use of tissue lysing machines is recommended instead of grinding the tissues manually by tube and pestle.

10.5. Diagnostic procedures

10.5.1. Overview

Apart from history, clinical symptoms and necropsy findings confirming the tissue lesions of the target organs (e.g. gills, kidney, spleen), additional diagnostic procedures may be employed in the field, such as quick Giemsa-stained spleen imprints or blood smears (characteristic bacteraemia). Isolation and identification may then follow.

A rapid fluorescent antibody (FA) technique, specific for *Ph.d.p.*, has been tested for early detection of the pathogen in fish farm waters prior to and subsequent to disease outbreaks among cultured fish (Mancuso *et al.*, 2013). Such a technique might prove useful to detect the "viable but not culturable" (VBNC) states of the bacterium early (Fouz *et al.,* 1998; Magarinos *et al.*, 1994; Pinto *et al.,* 2015) in water and sediments prompting prophylactic actions or aiding environmental surveys. This technique has shown experimentally that *Ph.d.p.* can be detected in water 20 days after the end of mortalities, proving that the pathogen is present in asymptomatic fish and is released into sea water for some time after an outbreak. The fluorescent antibody technique could be developed to distinguish inactive, active or damaged bacterial cell physiological states (Caruso *et al.*, 2003).

10.5.2. Primary cultivation of bacteria

Photobacterium damselae subspecies *piscicida* can be isolated by standard bacteriological sampling from trunk kidney, spleen, liver and/or brain and seeding on agar plates (usually TSA, blood or BHI agar). The brain is a suitable target tissue when fish are small, e.g. 2cm or less, after rinsing well with sterile saline solution.

Ph.d.p. grows well on standard Tryptone Soy Agar (TSA) with no need to supplement NaCl, since commercially available substrates contain 0.5% salt. Characteristic colonies appear after

about 24-36 hours of incubation at 22-28°C. These practical observations are in contrast to what is being described in the literature cited, where it is mentioned that the bacterium needs 1-2% NaCl supplementation of the substrates and takes 2-4 days of incubation to form its distinctive colonies (Romalde, 2002).

Photobacterium damselae subsp. *piscicida* -*Ph.d.p.*is a halophilic Gram-negative bacterium, appearing as a non-motile rod of 0.5 x 1.5 µm in size with bipolar staining and is pleomorphic in older cultures. Usually, bacterial cells shorten with age and acquire an ellipsoid shape. This "dwarfing" is compatible with cells entering the VBNC state (Magarinos *et al.,* 1994; Olivier, 2009).

10.5.3. Screening of pure cultures

Macroscopically *Ph.d.p.* colonies on the TSA medium are of characteristic morphology. They are smaller than or can measure up to 0.5mm in diameter. They are whitish (semi-translucent with irregular margins, like dewdrops, if observed under the light magnification of a stereoscope), somewhat viscous and adhere well to the substrate.

10.5.4. Identification of the strain

10.5.4.1. API

The interpretation of the API20E™ (bioMérieux) results via Apiweb™ does not identify *Photobacterium damselae* subsp. *piscicida,* because its profile is not contained in the bioMérieux code index. Nevertheless, this system is useful for the presumptive diagnosis of the pathogen, which produces the profile 2005004 almost invariably (Romalde, 2002). The phenotypic homogeneity of *Ph.d.p.* allows the use of this miniaturized system for its identification. Infrequently, however, the profile 0005004 has been produced (personal observation). Hence, this test should best be combined with other phenotypic characteristics, such as non-motility, negative urease test, no growth on thiosulfate citrate bile salts-sucrose (TCBS) agar as well as additional diagnostic methods, such as sero-agglutination and molecular tests.

Fig. 10.1. Biomerieux API 20E micro-tube test strip biochemical profile 2005004 identifying *Photobacterium damselae* subsp. *piscicida.* The typical score obtained on API 20E for *Ph.d.p.*

10.5.4.2. Slide agglutination

Photobacterium damselae subspecies *piscicida* -*Ph.d.p.* is serologically homogeneous, hence, serotypes have not been established (Bakopoulos *et al.*, 1997). The antigenic uniformity of lipopolysaccharide (LPS) profiles and outer membrane proteins (OMPs) encouraged the development of serological techniques for its detection and identification.

Commercial slide agglutination and latex agglutination test kits (Mono-Pp by Bionor AS), utilizing specific antiserum with polyclonal antibodies, may confirm the identification of the bacterium isolated on culture (Romalde *et al.*, 1995). No cross-reaction with other bacterial groups has been reported.

Nonetheless, in order to recognize minute serological differences among strains of disparate geographical regions, monoclonal instead of polyclonal antibodies (MAbs), that are specific against particular immunogens, may be utilized to identify *Ph.d.p.* intraspecific variations (Bakopoulos *et al*., 1997). Thus, for example, antigenic differences between Japanese and European strains have been revealed.

10.5.4.3. Mass spectrometry

Photobacterium damselae subspecies *damsela* and *piscicida* have important epizootiological and virulence differences. MALDI-TOF Mass Spectra biotyper analysis may correctly identify the species and discriminate the subspecies (Perez-Sancho *et al*., 2016) based on five differential peaks (m/z 4183 and 8367 for subsp. *damsela* and 4197, 8397 and 8856 for subsp. *piscicida*) using a genetic algorithm (ClinProTools software). This approach could be integrated into the workflow of laboratories possessing MALDI based tools for bacteria identification.

10.5.4.4. PCR

PCR may be utilized for both the confirmation of photobacteriosis and the screening for latent carriers among apparently healthy specimens. Several efforts to select appropriate PCR primer sets to discriminate between the closely related *Photobacterium damselae* subspecies, *Ph.d.p*. and *Ph.d.d*. have been published.

Developed PCR-based diagnostic methodologies are either multiplex, that is, they utilize sequences of different genes in addition to the 16S rRNA (e.g. the gene 1A coding for a penicillin-binding protein or the ureC gene, which is absent from *Ph.d.p*.), or are combined with other molecular techniques, such as AFLP or RFLP, or with plating the *Photobacteria spp.* on TCBS where only *Ph.d.d*. grows producing green colonies (Amagliani *et al*., 2009; Andreoni and Magnani, 2014; Essam *et al*., 2016, Osorio *et al*., 2000; Rajan *et al*., 2003; Zappulli *et al.,* 2005). Primer combinations are important in order to cope with the genotypic homogeneity between the *Photobacterium damselae* subspecies *damsela* and *piscicida*. Lately, a single-step real-time PCR assay, based on a bamB gene sequence (the gene responsible for the outer cell membrane protein assembly factor bamB) has proven sensitive and specific to discriminate between subspecies and quantify the existing genome copy numbers of the bacterium in infected fish tissue samples (Rajan *et al.,* 2005).

In order to bypass the need for prior isolation of the bacterium in pure culture and to overcome the 16S rRNA gene homogeneity between the *Ph.d*. subspecies, A PCR-RFLP method has been documented based on novel primer pairs designed on non-conserved sites of two genomic regions of several *Ph.d.p*. strains. These primers have been constructed subsequent to cloning and sequencing selected RAPD fragments and were found to be highly specific to *P. damselae* (Zappulli *et al*., 2005). In a second step, *Ph.d*. subspecies identification could be effected by restriction analysis of the PCR amplified products, which showed a unique digestion profile for all *Photobacterium damselae* subsp. *piscicida* strains tested. A distinctive RFLP pattern for *Ph.d.p*. allows the detection of this subspecies when *Ph.d.d*. is also present in the sample. This two-step method may be implemented directly on infected fish tissues, either from moribund fish or asymptomatic carriers.

Farm samples for PCR testing may include suspect fish, fertilized eggs, live prey, larvae/juveniles/fryor even sediments in order to reveal sub-clinical infection as well as moribund fish in order to confirm the diagnosis in case of overt disease. For this purpose, standardised, easy to use commercial qPCR kits have been made available from a number of companies.

The commercial PCR kits are designed for the in vitro quantitative detection of *Ph.d.p*. genomes and are designed to have the broadest detection profile possible, whilst remaining specific to the target bacterium genome. The primers and fluorogenic probe sequences in these kits are

proprietary and covered by patents and are advertized to have 100% homology with a broad range of *Ph.d.p*. sequences based on bioinformatics analyses.The qPCR kits provide copy number standard curves for the quantification of the amplified products and internal extraction template (DNA or RNA) controls for the quality of the nucleic acid (NA) extraction in order to eliminate false-negative results.

Subsequent to tissue lysing, NA extraction procedures with associated reagents are commercially available and most of them utilize spin column or magnetic bead technologies applied according to stepwise instructions.

Example of thermal cycles programmed into the PCR thermal cycler according to a commercial qPCR amplification kit for *Photobacterium damselae* subspecies *piscicida*, suggesting 50 thermal cycles:

Polymerase activation	Denaturation	Annealing - Extension - data collection
95°C	95°C	60°C
2 min	10 sec	60 sec
	50 cycles	

Example reaction mix and final volume in each well/micro-tube:

Mastermix	10 µl
Primer/probe mix	1 µl
RNAse/DNAse free water	4 µl
Reaction mix volume	15 µl
DNA template (sample NA extract)	5 µl
Final volume	20 µl

Primer sequences are proprietary and not disclosed.

However, laboratories may choose to develop their own protocol to detect *Ph.d.p.*, rather than use the commercial kits. Carraro *et al.* (2018) designed a highly sensitive real-time PCR assay for simultaneous detection and quantification of *P. damselae*subsp. *piscicida* and *P. damselae* subsp. *damsela* that was tested for specificity and sensitivity on laboratory-generated samples as well as on experimentally infected seabream tissue samples.

This assay targets a partial sequence of the bamB gene for amplification using specific primers PhPisc.B (For and Rev). Two single nucleotide polymorphisms in the target amplicon region determine two distinctive qPCR dissociation curves, so melting curve (dissociation) analysis can distinguish between *Ph.d.p. -Ph.d.d*.

Primers

Oligonucleotide	Sequence 5' → 3'
PhPisc B (For)	TGCTGGTGGTGTATTCTGGG
PhPisc B (Rev)	AACAGGTGTCGCATCAACGT

This assay can be performed using any colour-based chemistry and instrument that supports melting curve analysis. An example follows using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on LightCycler 480 System (Roche):

Reaction mix

Reagent	Final concentration/volume
PhPisc B (For)	10 uM
PhPisc B (Rev)	10 uM
Platinum SYBR Green Super-Mix-UDG	1X
DNA template	2.5
Water (molecular grade)	To 10 µl
Total volume	10 µl

Thermal profile

UDG incubation	Polymerase activation	Denaturation	Annealing/Extension	Dissociation
50°C	95°C	95°C	60°C	From 40°C to 95°C
2 min	2 min	10 sec	60 sec	at 4.4°C/s
			45 cycles	

Ph.d.p. strains should be characterized by melting temperature (Tm) of 83.3–84°C while *Ph.d.d.* strains should be characterized by a Tm of 84.3–84.9°C making the two subspecies distinguishable.

10.5.5. *In vitro* susceptibility testing

The most commonly used in vitro method to assess bacterium susceptibility to antimicrobials is the disc diffusion method (CLSI, 2011; Puttaswamy *et al.,* 2018). Although *Ph.d.p.* isolates may be distinguished from each other according to their antimicrobial susceptibility and even provide clues of their geographical origin (Bakopoulos *et al*., 1995; Thyssen and Olivier, 2001), it is evident in everyday practice that their sensitivity profile changes dynamically, depending on the degree of exposure of the bacterium to particular antibiotics/chemotherapeutics in the field (Smith, 2008). For example, regular use of a particular antibiotic on a farm renders it ineffective after about 3-4 treatment cycles.

By disc diffusion testing on TSA or MH agar plates (antibiogram), the pathogen is most often found sensitive to oxytetracycline, flumequine, oxolinic acid, florfenicol and potentiated sulphonamides (trimethoprim + sulfadiazine) and frequently resistant to ampicillin, amoxicillin, erythromycin. At times, however, amoxicillin shows potency.

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Photos

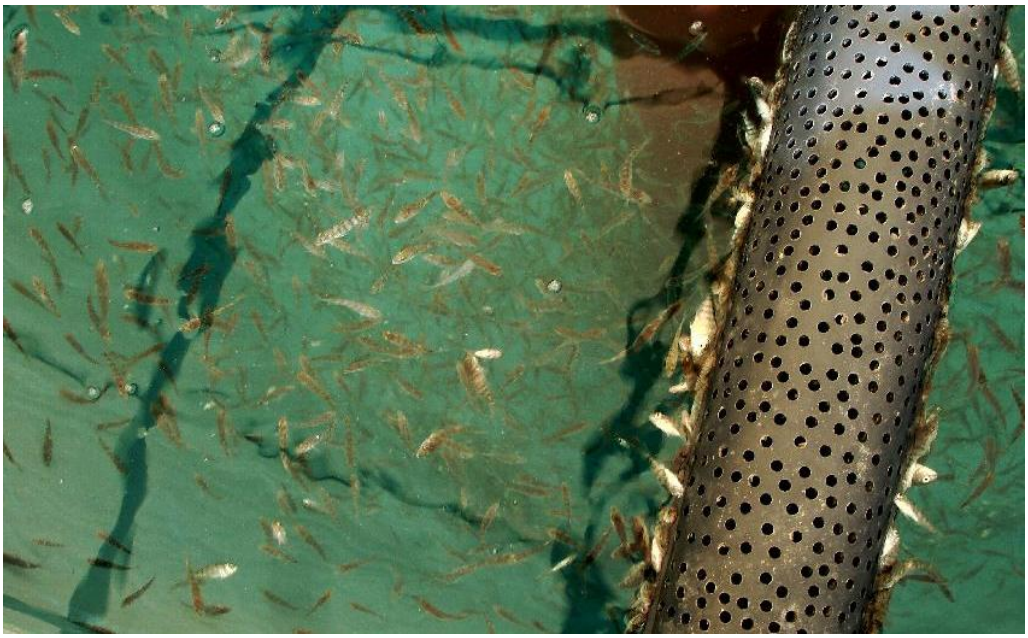

Fig. 10.2. Seabream fry (1 g) in nursery dying at the surface of their tank.

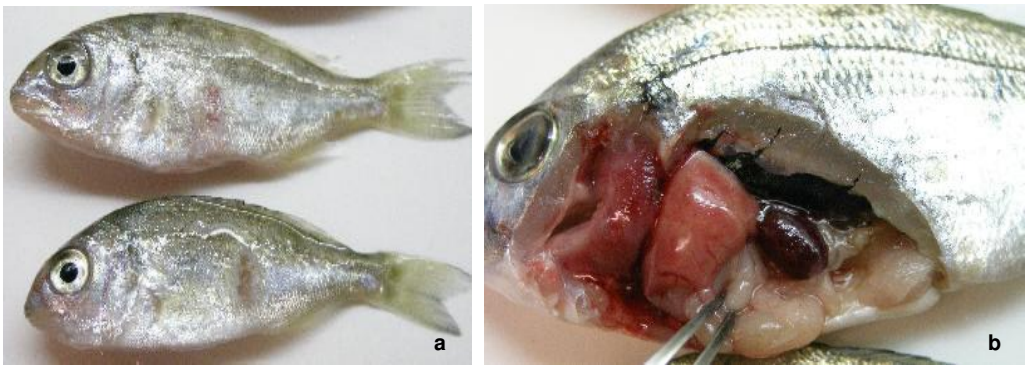

Fig. 10.3. a) Seabream fry (1 g) in nursery suffering photobacteriosis with skin lesions on the flanks; b) Mild liver congestion but gross spleenomegaly in bigger seabream (7 g).

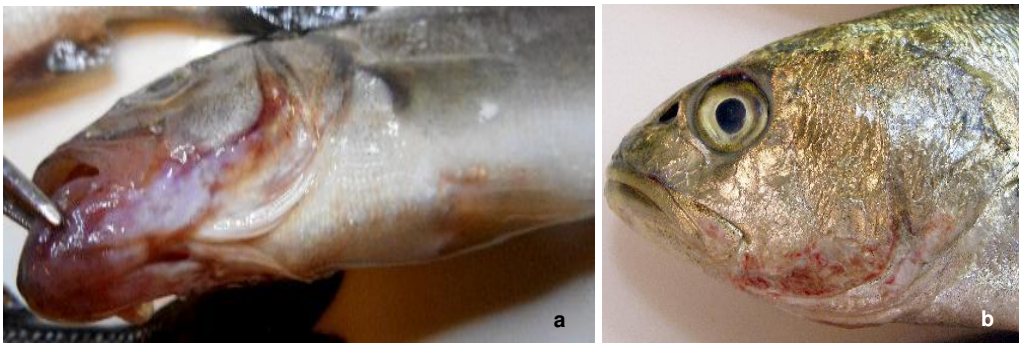

Fig. 10.4. a) Seabass weighing 30 g suffering photobacteriosis with external inflammatory lesions around the head epithelia, mainly lower jaw and opercula; b) Operculum skin with haemorrhagic inflammation on a meagre (200 g) infected by *Ph.d.p.*

Fig. 10.5. a) Seabass (50 g) with pale gills with large focal necrotic lesions; b) Inflamed and necrotic gill lamellae under light magnification 25 x.

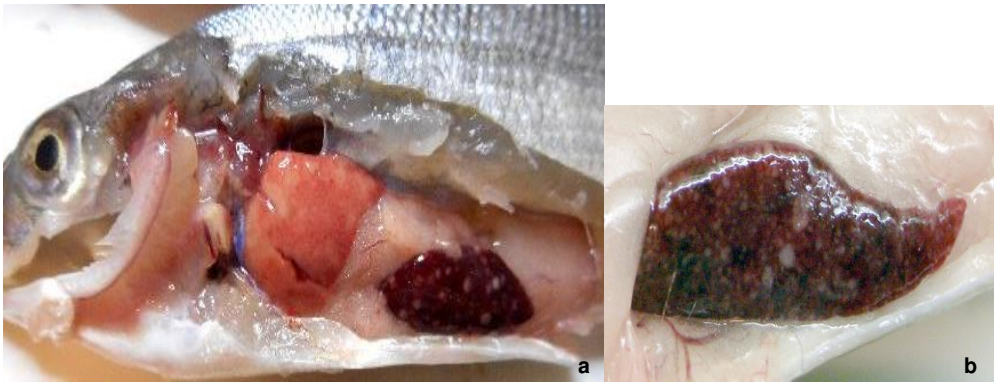

Fig. 10.6. a) Seabass (40 g) with pale gills, liver inflammation and gross splenomegaly with pseudotubercules; b) Close-up photo on seabass spleen with abundant pseudotubercules in the parenchyma.

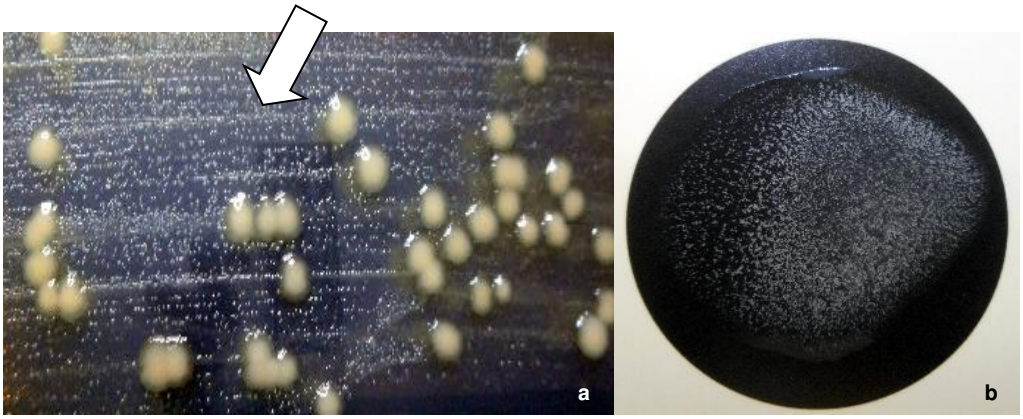

Fig. 10.7. a) Characteristic pin-point *Ph.d.p.* colonies on TSA agar, photographed against a black background for better contrast, after 24h incubation at room temperature (23oC). The larger 1.5mm colonies were produced by *Vibrio* spp., -mixed infection- but serve as a good comparison; b) Positive seroagglutination rapid test (Bionor Mono -Pp) confirming the pathogen.

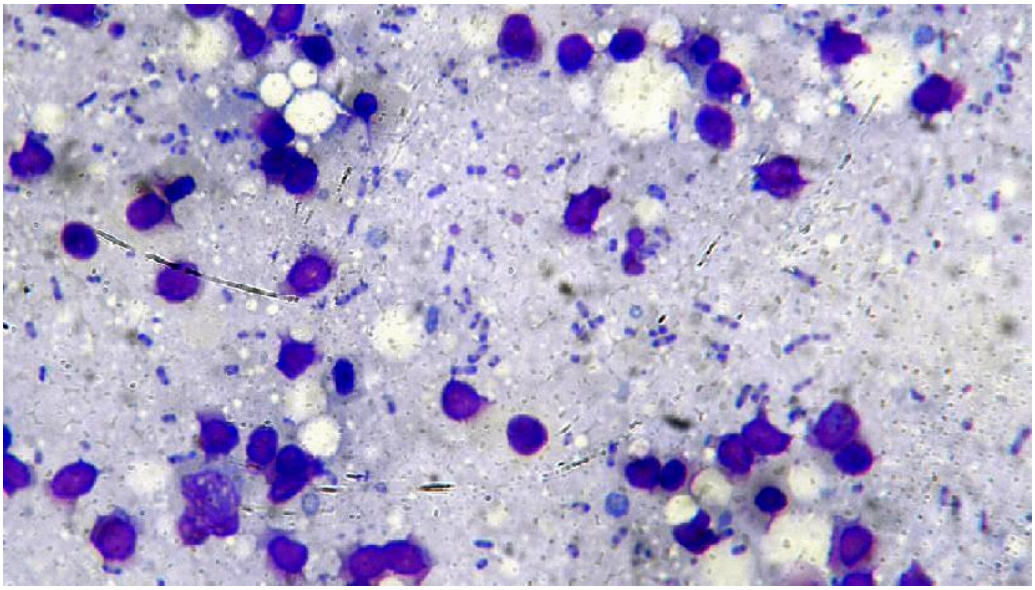

Fig. 10.8. Smear of homogenized splenic parenchyma from 70 day old seabream juveniles at the weaning section of a hatchery, fixed and stained with Giemsa. *Photobacteria* depicting characteristic biopolar staining were observed under 1000x magnification.

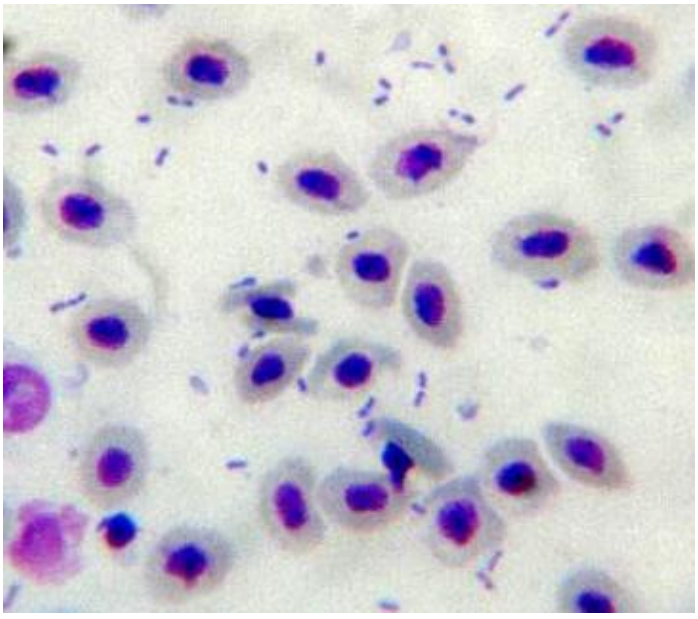

Fig. 10.9. Typical bacteraemia observed microscopically on Giemsa stained blood smear from seabass (17 g) suffering photobacteriosis.