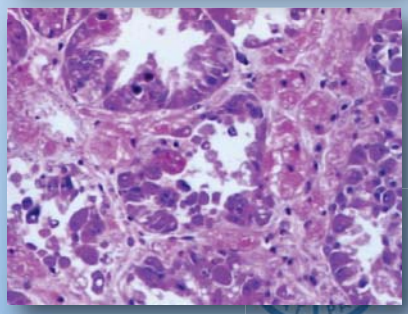
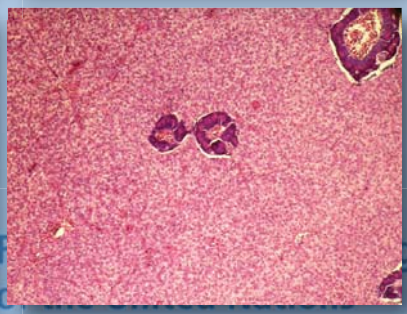
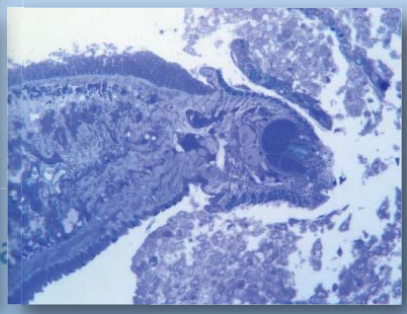
Food and Agriculture Organization of the United Nations



TCP/RER/3402

Assistance to Western Balkan Countries for Improving Compliance to International Standards

West Balkans Regional Aquatic Animal Disease Diagnostic Manual

The photographs in the cover were contributed by Prof. Olga Haenen, Prof. Ivona Mladineo and Dr. Snježana Zrnčić.

West Balkans Regional Aquatic Animal Disease Diagnostic Manual

by

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under

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Assistance to Western Balkan Countries for Improving Compliance to International Standards on Aquatic Animal Health

Food and Agriculture Organization of the United Nations
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Preparation of this document

The need for a coordinated regional approach to aquatic animal health and aquatic biosecurity was expressed and this goal realized through the Food and Agriculture Organization of the United Nations (FAO) Technical Cooperation Programme Project TCP/RER/3402 "*Assistance to Western Balkan Countries for Improving Compliance to International Standards on Aquatic Animal Health*". This regional project TCP/RER/3402 was based on a consultative and consensus-building process among countries of the West Balkan Region. It addresses the concerns expressed by the five participating FAO member countries in the region (i.e. Bosnia and Herzegovina, the Republic of Croatia, Republic of Macedonia, Montenegro and the Republic of Serbia), as having highest priority to assist all participating countries in developing capacity to implement international standards, i.e.: (i) risk analysis; (ii) disease surveillance, monitoring and reporting; (iii) disease diagnosis (including regional reference laboratories); and (iv) information and networking. The project's overall objective is to strengthen regional and national aquatic biosecurity governance and capacities for dealing with transboundary aquatic animal diseases (TAADs), and in the process improve compliance with international health standards for aquatic animals so that countries are better able to maintain and improve national aquatic animal health status, harmonize standards regionally, and better comply with the health standard requirements of regional and international trading partners.

This publication was conceived during the working group discussions that were carried out during the second regional workshop of the above project − regional workshop 2: "Improving Capacity for Diagnosis of Disease of Fish and Molluscs" held at Banja Luka, Bosnia and Herzegovina, from 20 to 24 October 2013 (See Annex 4).

This document represents one of the documentation outputs of the project TCP/RER/3402. It supports the FAO Strategic Objective 4 on Enable inclusive and efficient agricultural and food systems and Strategic Objective 5 on Increase the resilience of livelihoods to threats and crises. The finalization of this report was under the technical oversight of Dr Melba B. Reantaso, Aquaculture Officer, Aquaculture Branch (FIAA), Fisheries and Aquaculture Department of FAO and lead technical officer of TCP/RER/3402. Technical and English grammar editing was provided by Dr James Richard Arthur. All photographs were provided by Dr Snježana Zrnčić and Dr Vladimir Radosavljević unless otherwise indicated.

Abstract

This West Balkans Regional Aquatic Animal Disease Diagnostic Manual is a handbook whose main purpose is to facilitate the daily duties at aquaculture farms and provide a useful reference that will answer the majority of practical questions posed by official veterinarians, veterinary inspectors and fish health experts in five Western Balkan countries (Bosnia and Herzegovina, the Republic of Croatia, The former Yugoslav Republic of Macedonia, Montenegro and the Republic of Serbia). It is developed through the Food and Agriculture Organization of the United Nations (FAO) Technical Cooperation Programme Project TCP/RER/3402 "*Assistance to Western Balkan Countries for Improving Compliance to International Standards on Aquatic Animal Health*", a regional project that is based on a consultative and consensus-building process. This is a diagnostic guide for the surveillance, clinical inspection and sampling at aquaculture facilities with the aim to detect the diseases listed by the Word Organization for Animal Health (OIE) and the European Union (EU) according to their guidelines and standards, as well as other diseases of economic importance. Both standards include monitoring for diseases, the obligatory notification of clinical signs in registered farms and sampling by official veterinarians, activities that unconditionally require knowledge on diseases, farm production, normal appearance of the farmed species, and recognition of any changes that could lead to the suspicion of disease occurrence. Thus, this manual provides essential information on how to perform clinical inspections of fish and mollusc farms, how to recognize unusual behaviour of fish; how to select the most appropriate specimens for laboratory examination; and how to collect, pack and ship samples to the diagnostic laboratory. The laboratory procedures employed to identify the various disease agents are described, and information on the viral, bacterial and parasitic diseases of fish and molluscs in the Western Balkans is provided. The information presented should assist countries to maintain and improve their national aquatic animal health status, harmonize standards regionally, and better comply with the health standard requirements of regional and international trading partners.

(Key Words: Aquaculture, Aquatic Animal Diseases, Diagnostics, Guidelines, Health Standards, Notification, West Balkan)

Contents

Annexes

Acknowledgements

This publication was conceived during the working group discussions that were carried out during the regional workshop 2: "Improving Capacity for Diagnosis of Disease of Fish and Molluscs" and the contributions of all participants to this effort are sincerely acknowledged. Special thanks are extended to Dr Sanin Tanković, TCDC/TCCT consultant No. 1, Dr. Ivica Sučec, TCDC/TCCT consultant No. 4 and the National Project Coordinators (NPCs), Dr Svjetlana Nemet-Batinić of Bosnia and Herzegovina, Dr Vesna Petek of the Republic of Croatia, Dr Biljana Strojmanovska of The former Yugoslav Republic of Macedonia, Dr Tatjana Babović of Montenegro and Dr Tanja Labus of the Republic of Serbia, for their cooperation in providing us necessary information. Dr Melba Reantaso, lead technical officer of TCP/RER/3402 is gratefully acknowledged for her support, encouragement and fruitful discussions about the document and Dr Richard Arthur for his valuable suggestions during the editorial process. Thanks are offered to Dr Svjetlana Batinić of Bosnia and Herzegovina, Prof Dr Ivona Mladineo, Dr Branko Šoštarić and Dr Dražen Oraic of the Republic of Croatia, Dr Bruno Chollet of the French Republic, Dr Agus Sunarto of Indonesia, Dr Miwa Satoshi and Prof Kishio Hatai of Japan, Prof Dr Olga Haenen of the the Kingdom of the Netherlands, Prof Dr Vlasta Jenčič of the Republic of Slovenia, and FAO for providing photographs used in this manual. Mr Uwe Barg of FAO, the Italian Republic, is also thanked for helping provide information on Professor Fijan's activities on behalf of FAO. Special thanks are offered to Mrs Mirjana Fijan for providing his photograph.

Abbreviations and acronyms

0/129	Vibriostat (2,4-diamino 6,7-diisopropyl pteridine phosphate)
API	Analytical Profile Index (system)
BA	Blood agar
BCW	Bacterial coldwater disease
BF-2	Bluegill fin – permanent fibroblast cell line
BFNNV	Barfin flounder nervous necrosis virus
BHIA	Brain heart infusion agar
BKD	Bacterial kidney disease
CA	Competent authority
CCD	Charge-coupled device
CCV	Channel catfish virus
CE	Carp erythrodermatitis
CIFRI	Central Inland Fisheries Research Institute
CPE	Cytopathic effect
CT	Cycle threshold
CVI	Central Veterinary Institute
CyHV	Cyprinid herpesvirus
DNA	Deoxyribonucleic acid
EAFP	European Association of Fish Pathologists
EHN	Epizootic haematopoietic necrosis
EIFAC	European Inland Fisheries Advisory Commission
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimal essential medium
EPC	*Epithelioma papulosum cyprini* (epithelial permanent cell line)
ERM	Enteric redmouth disease
EU	European Union
EURL	European Reference Laboratory
EUS	Epizootic ulcerative syndrome
FAO	Food and Agriculture Organization of the United Nations
FARTC	Freshwater Aquaculture Research and Training Centre
FAT	Fluorescent antibody technique
FBS	Fetal bovine serum
FHM	Fathead minnow (epithelial permanent cell line)
FITC	Fluorescein isothiocyanate
FMM	*Flexibacter maritimum* medium
GP	Glucose peptone agar or medium
GPY	Glucose peptone yeast agar or medium
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
HRP	Horseradish peroxidase
IFAT	Indirect fluorescent antibody test
IHN	Infectious haematopoietic necrosis
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anemia
ISH	*In situ* hybridization
ITS	Internal transcribed spacer (region)
KDM-2	Kidney disease medium
KHV	Koi herpesvirus
KHVD	Koi herpesvirus disease
LAMP	Loop mediated isothermal amplification
LFA	Laminar flow apparatus

MA	Marine agar
MAS	Motile aeromonad septicaemia
MHA	Mueller-Hinton agar
MIC	Minimal inhibitory concentration
mRNA	Messenger ribonucleic acid
NA	Nutrient agar
NVI, DTU	National Veterinary Institute, Danish Technical University
NVL	National Veterinary Laboratory
O-F	Oxidation-fermentation (test)
OIE	Word Organisation for Animal Health
OPD	Ortho-phenylenediamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT-qPCR	Real-time reverse transcription polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RGNNV	Redspotted grouper nervous necrosis virus
RNA	Ribonucleic acid
RSID	Red seabream iridoviral disease
RTFS	Rainbow trout fry syndrome
RTG-2	Rainbow trout gonads (fibroblast permanent cell line)
RT-PCR	Reverse transcription-polymerase chain reaction
RVC	*Rhabdovirus carpio*
SAV	Salmonid alphavirus
SJNNV	Striped jack nervous necrosis virus
SKDM	Selective kidney disease medium
SSN-2	Striped snakehead (*Channa striata*) permanent cell line
SVC	Spring viraemia of carp
SVCV	Spring viraemia of carp virus
TAADs	Transboundary aquatic animal diseases
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
TCP	Technical Cooperation Programme (of the FAO)
TNNV	Turbot nervous necrosis virus
TPNNV	Tiger puffer nervous necrosis virus
TRIS	Tris(hydroxymethyl) aminomethane
TSA	Trypticase soy agar
UK	United Kingdom of Great Britain and Northern Ireland
UV	Ultraviolet
VER	Viral encephalopathy and retinopathy
VHS	Viral haemorrhagic septicaemia
VN	Virus neutralization (test)
VNN	Viral nervous necrosis
WHO	World Health Organization

Glossary[1]

Acquired immunity	a defence response developed following recovery from an infection (or vaccination) by a specific infectious agent (or group of agents)
Aetiology	the study of the transmission and infectivity of an aetiologic agent
Anaemia	a deficiency in blood or of red blood cells
Anorexia	loss of appetite
Antibody	a protein capable of cross-reacting with an antigen
Antigen	a substance or cell that elicits an immune reaction
Antimicrobial agent	any substance of natural, semisynthetic or synthetic origin that at in vivo concentrations kills or inhibits the growth of micro-organisms by interacting with a specific target
Aquatic animals	live fish, molluscs and crustaceans, including their reproductive products, fertilized eggs, embryos and juvenile stages, whether from aquaculture sites or from the wild
Ascites	accumulation of serous fluid in the abdominal cavity (also known as "dropsy")
Atrophy	decrease in the amount of tissue, or size of an organ, after normal growth has been achieved
Autolysis	enzyme-induced rupture of cell membranes, either as a normal function of cell replacement or due to infection
Bacteriology	branch of science that deals with the study of bacteria
Bacterium (bacteria)	a unicellular prokaryotic (nuclear material not contained within a nucleus) microorganism that multiplies by cell division (fission), typically has a cell wall, and which may be aerobic or anaerobic, motile or non-motile, free living, saprophytic or pathogenic
Basophilic	acidic cell and tissue components staining readily with basic dyes (e.g. hematoxylin); chromatin and some secretory products in stained cells appear blue to purple
Biosecurity	the sum total of the activities and measures taken by a region, country, group of aquaculture producers or single aquaculture production facility to protect its natural aquatic resources, capture fisheries, aquaculture, biodiversity and/or cultured stocks and the people who depend on them from the possible negative impacts resulting from the introduction and spread of serious aquatic animal diseases
Broodstock	sexually mature fish, molluscs or crustaceans
Carrier	an individual who harbors the specific organisms causing a disease without manifest clinical signs and which is capable of transmitting the infection; the condition of such an individual is referred to as "carrier state"
Cachetic	having cachexia; wasting away from a disease or chronic illness
Chromatin	nucleoprotein complex containing genomic DNA and RNA in the nucleus of most eukaryotic cells
Chronic	a long-term infection which may or may not manifest clinical signs
Contagious	a disease normally transmitted only by direct contact between infected and uninfected organisms
Cyst	a resilient dormant stage of a free-living or parasitic organism
Cytology	the study of cells, their origin, structure, function and pathology
Cytopathic effect	pertaining to or characterized by pathological changes in cells
Deoxyribovirus (DNA-virus)	a virus with a deoxyribonucleic acid genome (cf. ribovirus)

[1]Most definitions used in this glossary are taken from OIE *Aquatic Animal Health Code* and *Manual of Diagnostic Tests for Aquatic Animals* (available at: www.oie.int) or from other FAO publications

Disease	any deviation from or interruption of the normal structure or function of any part, organ or system (or combination thereof) of the body that is manifested by a characteristic set of clincal signs and whose aetiology, pathology and prognosis may be known or unknown
Disease agent	an organism that causes or contributes to the development of a disease
Diagnosis	determination of the causative agent of a disease
Disinfection	the application, after thorough cleansing, of procedures intended to destroy the infectious or parasitic agents of diseases of aquatic animals; this applies to aquaculture establishments (e.g. hatcheries, fish farms) and to objects that may have been directly or indirectly contaminated
Deoxyribonucelic acid	nucleic acid containing the bases adenine, guanine, cytosine and thymine. Single-strand DNA (ssDNA) occurs in some viruses (usually as a closed circle). In eukaryotes and many viruses, DNA is double-stranded (dsDNA)
Deoxyribonucelic acid (DNA) probes	segments of DNA labelled to indicate detection of homologous segments of DNA in samples of tissues or cultures (cf. RNA probes)
Diapedesis	the movement or passage of blood cells, especially white blood cells, through intact capillary walls into surrounding body tissue
Ectoparasite	a parasite that lives on the external surface of the host
Enzyme-linked immunosorbent assay	an assay used to detect antigen (antigen capture ELISA) or antibody (antibody capture ELISA)
Emaciation	a wasted condition of the body
Endothelium	the layer of epithelial cells originating from the mesoderm that lines the cavities of the heart, the blood and lymph vessels, and the serous cavities of the body
Enophthalmia	sunken eyes
Envelope	in virology, the lipoprotein membrane composed of host lipids and viral proteins (non-enveloped viruses are composed solely of the capsid and nucleoprotein core)
Enzootic	present in a population of animals at all times but, occurring only in small numbers of cases
Eosinophilic	basic cell and tissue components staining readily with acidic dyes (e.g. eosin); stained cells appear pink to red
Epithelium	the layer of cells covering the surface of the body and comprising all gastrointestinal linings
Epitope	the structural component of an antigen which stimulates an immune response and which binds with antibody
Epizootic	affecting many animals within a given area at the same time; widely diffused and rapidly spreading
Epizootiology	the study of factors influencing infection of animal populations by a pathogenic agent
Erosion	destruction of the surface of a tissue, material or structure
Eukaryote	an organism having chromosomes that are contained within a membrane-bound nucleus (cf. prokaryote)
Exopthalmia	abnormal protrusion of the eyeballs (also known as “popeye”)
Exudate	material, such as fluid, cells or cellular debris, which has escaped from blood vessels and has been deposited in tissues or on tissue surfaces, usually as a result of inflammation
Filtration	passage of a liquid through a filter, accomplished by gravity, pressure or vacuum (suction)
Fixation	preservation of tissues in a liquid that prevents protein and lipid breakdown and necrosis; the specimen is hardened to withstand further processing, and the cellular and subcellular contents are preserved in a manner close to that of the living state

Fixative	a fluid (e.g. an aldehyde or ethanol-based solution) that prevents denaturation and autolysis by cross-linking of proteins
Foreign body	any organism or abiotic particle not formed from host tissue
Formalin	a 37 percent solution of formaldehyde gas
Fungus	any member of the Kingdom Fungi, comprising single-celled or multinucleate organisms that live by decomposing and absorbing the organic material in which they grow
Furuncles	raised liquefactive muscle lesions
Gram's stain	a stain used to differentiate bacteria with permeable cells walls (Gram-negative) and less-permeable cell walls (Gram-positive)
Granuloma	any small nodular-delimited aggregation of granular haemocytes or modified macrophages resembling epithelial cells (epithelioid cells)
Granulomatosis	any condition characterized by the formation of multiple granulomas
Gross signs	signs of disease visible to the naked eye
Haematopoietic	pertaining to or effecting the formation of blood cells
Haemocytes	blood cells
Haemolymph	the cell-free fraction of the invertebrates' blood containing a solution of protein and non-proteinaceous defensive molecules
Haemorrhage	escape of blood from the vessels
Hepatopancreas	digestive organ composed of ciliated ducts and blind-ending tubules which secrete digestive enzymes for uptake across the digestive tubule epithelium; also responsible for release of metabolic by-products and other molecular or microbial wastes
Histology	the study that deals with the minute structure, composition and function of tissues
Histopathology	structural and functional changes in tissues and organs which are seen in samples processed by histology that cause or are caused by a disease
Host	an individual organism that is infected by another organism
Hyperplasia	abnormal increase in size of a tissue or organ due to an increase in number of cells
Hypertrophy	abnormal enlargement of cells due to irritation or infection by an intracellular organism
Hyphae	tubular cells of filamentous fungi; may be divided by crosswalls (septae) into multicellular hyphae; may be branched; interconnecting hyphae are called mycelia
Icosahedral	shape of viruses with a 5-3-2 symmetry and 20, approximately equilateral, triangular faces
Immunity	protection against infectious disease conferred either by the immune response generated by immunization or previous infection or by other non-immunologic factors
Immunization	protection against disease by deliberate exposure to pathogen antigens to induce defence system recognition and enhance subsequent responses to exposure to the same antigens (syn.: vaccination)
Immunofluorescence	any immuno-histochemical method using antibody labeled with a fluorescent dye. Direct if a specific antibody or antiserum with a fluorochrome and used as a specific fluorescent stain. Indirect if the fluorochrome is attached to an antiglobulin, and a tissue constituent is stained using an unlabeled specific antibody and the labeled antiglobulin, which binds the unlabeled antibody
Immunoglobulin	a family of proteins constructed of light and heavy molecular weight chains linked by disulphide bonds; usually produced in response to antigenic stimulation
Immunization	induction of immunity
Indirect fluorescent antibody technique (IFAT)	a technique using unlabelled antibody and a labelled anti-immunoglobulin to form a "sandwich" with any antigen-bound antibody
Infectious	capable of being transmitted or of causing infection

Infiltration	in invertebrates, haemocyte migration to a site of tissue damage or infection by a foreign body or organism (syn.: "inflammation")
Inflammation	in invertebrates, an infiltration response to tissue damage or a foreign body; the infiltration may be focal, diffuse or systemic (syn.: infiltration)
Innate immunity	a host defence mechanism that does not require prior exposure to the pathogen
Intercellular	situated or occurring between the cells in a tissue
Lepidorthosis	erection of scales caused by disease or parasites
Lesion	any pathological or traumatic change in tissue form or function
Lethargy	abnormal drowsiness or stupor (response only to vigorous stimulation); a condition of indifference
Macrophages	in vertebrates, large (10–20 mm) amoeboid blood cells, responsible for phagocytosis, inflammation, antibody and cytotoxin production
Micro-organism	principally, viruses, bacteria and fungi (microscopic species, and taxonomically related macroscopic species); Microscopic protistans and algae may also be referred to as micro-oorganisms
Molecular probes	see DNA probes
Monoclonal antibodies	referring to identical antibody molecules produced by clonage of the antibody-producing cell and responsive to a single antigen epitope
Moribund	near death
Mucus	the free slime of the mucous membrane, composed of secretion of the glands, along with various inorganic salts, desquamated cells and leukocytes
Necrosis	sum of the morphological changes indicative of cell death and caused by the progressive and irreversible degradative action of enzymes
Notifiable diseases	(a) Notifiable to the OIE means the list of transmissible diseases considered to be of socio-economic and/or public health importance within countries and that are significant in the international trade in aquatic animals and aquatic animal products. (b) Notifiable to the EU means those fish diseases that are notifiable according to EU legislation (i.e. Council Directive 2006/88/EC−on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals)
Oedema	the presence of abnormally large amounts of fluid in the intercellular spaces
Outbreak	the sudden onset of disease in epizootic proportions
Parasite	an organism which lives upon or within another living organism (the host) at whose expense it obtains some advantage, generally nourishment
Passage	the successive transfer of a virus or other infectious agent through a series of experimental animals, tissue culture or synthetic media with growth occurring in each medium
Pathogen	an infectious agent capable of causing disease
Pathogenicity	the ability to produce pathologic changes or disease
Pathology	the branch of science dealing with the essential nature of disease, especially of the structural and functional changes in tissues and organs of the body which cause or are caused by a disease
Phagocytosis	uptake by a cell of material from the environment by invagination of its plasma membrane
Polyclonal antibodies	collection of immunoglobulins secreted by different B cell lineages within the body, whereas monoclonal antibodies come from a single cell lineage but they react against a specific antigen, each identifying a different epitope
Polymerase chain reaction	a process by which nucleic acid sequences can be replicated ("nucleic acid amplification")
Pop-eye	abnormal protrusion of the eyes from the eye sockets (syn.: exophthalmia)

Prevalence	the percentage of individuals in a sample infected by a specific disease, parasite or other organism
Prokaryote	cellular micro-organisms in which the chromosomes are not inclosed within a nucleus
Prophylactic (-axis)	an action or chemotherpeutant administered to healthy animals in order to prevent infection (cf. treatment)
Pustule	a subepidermal swelling containing necrotic cell debris as a result of inflammation (haemocytic infiltration) in response to a focal infection
Quarantine	the holding or rearing of aquatic animals under conditions which prevent their escape, and the escape of any pathogens they may be carrying, into the surrounding environment; this usually involves sterilization of all effluent and quarantine materials
Reservoir (host or infection)	an alternate or passive host or carrier that harbors pathogenic organisms without injury to itself, and serves as a source from which other individuals can be infected
Resistance	(a) to disease – the capacity of an organism to control the pathogenic effects of an infection (cf. acquired immunity and innate immunity); (b) antibiotic or "drug" resistance – the capability of a microbe to evade destruction by an antibiotic
Ribonucleic acid (RNA)	nucleic acid consisting of ribonucleotides made up of the bases adenine, guanine, cytosine and uracil
Ribonucleic acid (RNA) probes	segments of RNA which are labelled to detect homologous segments of RNA or DNA in tissue or culture samples
Septicaemia	systemic disease associated with the presence and persistence of pathogenic micro-organisms or their toxins in the blood (syn.: blood poisoning)
Spore	the infective stage of an organism that is usually protected from the environment by one or more protective membranes (syn.: zoospore)
Stress	the sum of biological reactions to any adverse stimuli (physical, internal or external) that disturb the organism's optimal operating status
Stroma	the supportive framework of an organ (or gland or other structure), usually composed of connective tissue
Surveillance	a systematic series of investigations of a given population of aquatic animals to detect the occurrence of disease for control purposes, and which may involve testing of samples of a population
Susceptible	an organism which has no immunity or resistance to infection by a another organism
Syndrome	an assembly of clinical signs which when manifest together are indicative of a distinct disease or abnormality (syn.: pathognomic/pathognomonic)
Systemic infection	an infection involving the whole body
Transmission	transfer of an infectious agent from one organism to another; transmission may be "horizontal" (i.e. direct from the environment (e.g. via ingestion, skin and gills) or "vertical" (i.e. passed from parent to egg, either inside the egg (intra-ovum) or through external exposure to pathogens from the parent generation)
Trauma	an effect of physical shock or injury
Treatment	an action taken to eradicate an infection (cf. prophylaxis)
Tumour	abnormal growth as a result of uncontrolled cell division of a localized group of cells
Ubiquitous	existing or being everywhere
Ulcer	an excavation of the surface of an organ or tissue, involving sloughing of necrotic inflammatory tissue
Vaccine	an antigen preparation from whole or extracted parts of an infectious organism, which is used to enhance the specific immune response of a susceptible host

Virulence	the degree of pathogenicity caused by an infectious organism, as indicated by the severity of the disease produced and its ability to invade the tissues of the host; the competence of any infectious agent to produce pathologic effects; virulence is measured experimentally by the median lethal dose (LD50) or median infective dose (ID50)
Virus	one of a group of minute infectious agents, characterized by a lack of independent metabolism and by the ability to replicate only within living host cells

Homage to Professor Nikola Fijan

It is impossible to complete this *West Balkans Regional Aquatic Animal Disease Diagnostic Manual* without mentioning Professor Nikola Fijan, a pioneer of ichthyopathology in this region. He became a world-renowned expert as the creator of the *Epithelioma papulosum cyprini* (EPC) permanent cell line, suitable for the propagation of the majority of freshwater fish viruses, and as the first scientist to isolate the viruses causing spring viraemia of carp (SVC) and channel catfish virus (CCV) disease.

Professor Fijan spent his early childhood at a fish farm which was managed by his father. Probably there he realized the desire to work in fisheries. Therefore, he studied veterinary medicine and directed his studies towards those disciplines related to fish biology, physiology and pathology. After graduation, he became professionally engaged by the Department for Biology and Pathology of Fish and Bees at the Veterinary Faculty of the University of Zagreb, the Republic of Croatia, where he was awarded his Ph.D. degree.

In addition to his exceptional capabilities in science, he was a great teacher, mentoring numerous Master of Sciences and Doctor of Philosophy candidates in the field of aquaculture from the entire territory of The former Yugoslavia, as well as from other countries. He was also actively involved in establishing diagnostic laboratories servicing the aquaculture sector.

Besides playing important roles in international organizations such as the World Health Organization (WHO) and the World Organisation for Animal Health (OIE), in the 1970s to 1990s, Professor Fijan worked with the European Inland Fisheries Advisory Commission (EIFAC) of the Food and Agriculture Organization of the United Nations (FAO) on numerous tasks. He was Chairman of EIFAC's Sub-Commission II - Fish Culture and Fish Diseases and convener of various working parties (e.g. on veterinary drugs used in pond aquaculture, and on withdrawal periods of drugs used in aquaculture). He worked as expert in many FAO projects such as the "Intensification of Freshwater Fish Culture and Training Project" (FI:DP/IND/75/031) which was carried out at the Freshwater Aquaculture Research and Training Centre (FARTC) of the Central Inland Fisheries Research Institute (CIFRI) at Dhauli, Bhubaneswar, India, and he also worked in the Latin American Regional Aquaculture Centre in Pirassununga, Brazil.

As an aquaculture and fish health expert, Professor Fijan worked on improvement of product quality in pond culture, intensification of freshwater fish culture, spawning and larval rearing of catfish, disease prevention

and control, and the implementation of prophylactic measures in European and Asian countries and in Brazil. Twice, he worked in the United States of America as a visiting professor, at Auburn University, Auburn, Alabama and at the University of Arkansas, Pine Bluff, Arkansas.

He published many scientific papers, but what is most important to highlight in his career is that he was such a generous teacher, always supporting and guiding his younger colleagues. He fostered our creativity and scientific curiosity. It did not matter what he worked on in his office, when younger colleagues had any question, he was ready to discuss with them and to find answers to their questions. It was a privilege to work with him both in the laboratory and in the field, on the fish farm. After retirement, he was still enthusiastic and full of creativity that he readily shared with younger colleagues. We enjoyed his visits and lively discussions, not merely about fish and molluscan diseases but about all current topics. Unfortunately, a severe disease took him away on 29 July 2009. Below, I'd like to cite just a few notes received from people who knew him.

Snježana Zrnčić

"I remember him from the Dubrovnik meeting in 1986, as a very warm-hearted, generous, wise, and father-like person in our field of work. He has published very important research on SVC, carp erythrodermatitis and other subjects, from which we benefit still in our work. He will be remembered by many of us."

Dr.ir. Olga L.M. Haenen
Head of the Fish and Shellfish Diseases Laboratory
Central Veterinary Institute (CVI) of Wageningen
Lelystad

"He was a lovely giant of a man and will always be remembered by those who were fortunate to meet him. I first met him over 30 years ago and he was involved with me in setting up the EAFP. I will be giving a talk at the EAFP General Assembly in Prague about the way the EAFP has developed over the years since it was established so it will be appropriate for me to include an acknowledgement to Nicola's contribution to the Association but also to our knowledge of fish diseases over many years.The sadness will pass away with time and leave happy memories of him."

Dr Barry Hill
Retired Aquatic Animal Health Expert

"It is with great sadness to learn that our good colleague, friend and long-term collaborator passed away. I do remember Professor Fijan very well and also recall many of the highly inspiring and fruitful discussions on fish health and aquaculture development. We shared many common objectives and opinions dealing with aquaculture and fish health as well as environmental management. His departure is not only a big loss for you and your institution but also for all of us in the international science community who worked with him.

Harald Rosenthal
University of Kiel
President, World Sturgeon Conservation Society

"We will remember Nikola Fijan as a highly competent and dedicated scientist and world- reknown expert. He contributed to numerous FAO projects and initiatives. We owe him respect and gratitude for his work and friendship."

Uwe Barg, Aquaculture Officer
Fisheries and Aquaculture Department
Food and Agriculture Organization of the United Nations
Rome, The Italian Republic

1. Introduction

Western Balkan countries are situated in an area with favourable conditions for aquaculture development. The tradition of mollusc cultivation dates back to the Roman times, and freshwater aquaculture has a history older than a century, while the culture of Mediterranean fish species in marine cages started in the 1970s, concurrently with similar projects in the French Republic and the Italian Republic. There are many waterbodies suitable for the establishment of new aquaculture farms or for the enhancement of production on existing ones. Expansion or intensification of aquaculture production is accompanied by the risk of disease introduction and spread, and thus the establishment of preventive measures is inevitable. Several countries in the region share rivers or watersheds, and three of them (Bosnia and Herzegovina, the Republic of Croatia and Montenegro) are connected by the Adriatic Sea, creating integrated epidemiological areas.

In contrast to terrestrial animal production, aquaculture is highly dynamic and characterized by an enormous diversity of farmed species and environmental conditions. Thus salmonids are cultured in cold freshwater or brackishwater areas and the production system may involve earthen ponds, raceways, cages, etc. Warmwater freshwater fish species such as carps and catfishes are usually cultured in earthen ponds having conditions similar to those occurring in lakes, but are sometimes also reared in cages; water temperatures may range from freezing at the water surface during winter to up to 35°C during summer months. Mariculture includes the culture of gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and similar species in marine cages, and the raft culture of molluscs. Aquaculture operations in the region vary from small-scale, traditional family farms to sophisticated industrial operations.

All five countries (Bosnia and Herzegovina, the Republic of Croatia, Montenegro, The former Yugoslav Republic of Macedonia and the Republic of Serbia) involved in the FAO Technical Cooperation Programme Project TCP/RER/3402 "*Assistance to Western Balkan Countries for Improving Compliance to International Standards*" are aiming to market their aquaculture products to the common European Union (EU) market, as either EU member countries, candidate countries, or simply as third-country trading partners. However, the free movement of aquaculture products can facilitate the spread of devastating diseases which can threaten national aquaculture industries. To prevent such adverse events, it is important to develop and implement necessary mechanisms for the early detection, identification and elimination of infected animals from individual aquaculture facilities. The best way to achieve this is to put in place a good disease surveillance programme. Targeted surveillance with the aim of demonstrating national freedom from disease should be based on a comprehensive knowledge of the particular disease being considered. Surveillance activities should be in accordance with the guidelines and standards of the World Organisation for Animal Health (OIE) and the EU. The OIE *Aquatic Animal Health Code* sets out standards for the improvement of aquatic animal health and welfare of farmed fish worldwide, including through standards for safe international trade in aquatic animals and their products. The objective of *Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals* (see Anon., 2006) is to raise standards of aquaculture health throughout the EU and to control the spread of disease while maintaining the freedom to trade. Both standards include monitoring for disease and the obligatory notification of clinical signs in registered farms and sampling by official veterinarians. These activities are tasks that unconditionally require knowledge on diseases, as well as knowledge about farm production, the normal appearance of the farmed species, and recognition of any changes that could lead to the suspicion of disease occurrence.

Moreover, it is necessary to know how to perform clinical inspections of the fish or mollusc farms; the topics that must be included during on-farm inspection; how to recognize unusual behaviour of fish; how to select which specimens are the most appropriate for laboratory examination; and how to collect, pack and deliver samples to the diagnostic laboratory. Thus the main purpose of this manual is to facilitate the veterinary inspectors' daily duties at the aquaculture farms and to provide them with a useful manual that will answer the majority of their practical questions.

Regardless of differences in culture technology, all epidemiologically connected aquaculture facilities face the same pathological threats. A basic knowledge of aquaculture systems and the characteristics of the important diseases of cultured aquatic animals is also requisite for involvement of aquatic animal disease

experts in the hazard identification and risk assessment portions of pathogen risk analysis, regardless of whether the risk analysis being undertaken is due to the proposed movement of live aquatic animals (e.g. the potential introduction of exotic pathogens (including TAADs) or of new strains of existing pathogens) or due to the possible biomagnification of existing pathogens present at aquaculture establishments or in wild populations.

Once a country has implemented targeted surveillance and achieved disease-free status, the introduction of a new disease may cause significant losses. In order to diminish losses, the competent authority (CA) responsible for aquatic animal health should prepare contingency plans for quick action in the event of a disease outbreak. Contingency plans should be developed for individual diseases and be based on the diagnostic procedures for the disease under consideration, and all needed actions should be based on a knowledge and understanding of the disease pattern. Therefore, this manual should also be a useful reference for the creation of contingency plans for listed diseases.

The manual is designed as follows:

- The Introduction informs the reader about the diversity of aquaculture activities in the West Balkan Region and the importance of disease surveillance and control as a prerequisite for successful and profitable business.
- Section 2 presents basic data on aquaculture facilities to facilitate inspection of fish and mollusc farms, followed by the methods of sample selection.
- Section 3 provides instructions for preserving, packing and shipping live fish, fresh fish and molluscs and their organs in transport media or fixatives to the disease diagnostic laboratory.
- Section 4 describes the different diagnostic techniques employed to identify the disease agent and to decide about antibacterial treatment, such as external examination, necropsy, cytology, bacteriology, virology and histology, as well as molecular techniques.
- Section 5 provides information about viral diseases, with priority given to several EU non-exotic listed diseases: viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN) and koi herpesvirus disease (KHVD). However, diseases of regional concern with economic impact to the local aquaculture industry are also included, (e.g. spring viraemia of carp (SVC), infectious pancreatic necrosis (IPN) and viral encephalopathy and retinopathy (VER).
- Section 6 includes bacterial diseases; because they are often confused with certain viral diseases, veterinarians should also have a basic knowledge about them for use during evaluation of the health status of aquaculture facilities. The other diseases described in this section occur on a regular basis at farms in the region and are the cause of economic losses.
- Section 7 acquaints the reader with epizootic ulcerative syndrome (EUS), a disease which represents a potential threat to cyprinid aquaculture, as well as to species in open waters, with huge range of susceptible species and a wide geographic distribution.
- Section 8 includes two OIE and EU-listed diseases of molluscs: bonamiosis and marteiliosis. European flat oyster (*Ostrea edulis*) cultured along the Adriatic coast are susceptible to both diseases, while the Mediterranean mussel (*Mytilus galloprovincialis*) is susceptible to marteiliosis.
- Annex 1 provides information on the preparation of several basic formulations (fixatives, culture media) needed for the preservation of tissues for virology, for histology and for the primary isolation of bacteria. A list of antimicrobials approved for use in fish in several countries of the region is also included as Annex 2.

It is hoped that this manual will assist official veterinarians and fish health specialists to perform their duties, as well as serve as a source of useful information to all those involved in aquatic animal health management, and finally, that it will help to improve national aquaculture production among the countries of the West Balkan Region.

2. Sampling of fish for diagnostic purposes

2.1 On-site inspection

An inspection of the aquaculture facility by a qualified fish health specialist is the first step in the evaluation of the health status of the cultured aquatic animals. There are several activities that should be included in the inspection:

- Review of data recorded on the farm by personnel:
  - records of environmental and production data (i.e. temperature, oxygen saturation, weather conditions, salinity, water transparency, stocking density, appetite and feed consumption);
  - registered mortality rate per culture unit;
  - notes on visible signs of disease and abnormal behaviour;
  - notes on movement of fish or gametes inside the farm, introduction from other farms or shipment to other farms;
  - information on previous disease outbreaks, diagnostic reports and treatment; and
  - information on disinfection and biosecurity measures implemented.

- Inspection of all units with fish, preferably before daily removal of any sick or dead fish, with the aim of observing any weak fish, abnormal behaviour or signs of disease. According to the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2014), relevant European and certain national legal acts, there are different approaches to the inspection and sampling of aquaculture farms:

  - *Sampling of fish for routine monitoring/surveillance for evaluation of health status of the population* – Surveillance is elaborated in detail in the "*Surveillance manual for regional approach to surveillance of VHS/IHN, SVC and KHV"* for several of the most important diseases in the Western Balkan Region and in several of the following sections, sampling as a part of surveillance for the monitoring of particular aquaculture commodities will be described in detail.

  - *Sampling for issuing a health certificate* – Sampling to demonstrate freedom from disease should include selection of samples in a manner that increases the chance of detecting the pathogen and the use of the most sensitive diagnostic method available.

  - *Sampling for investigation of a disease outbreak/increased mortalities at an aquaculture facility* – The fish farmer/owner is obliged to notify the CA of any abnormal mortality or disease outbreak, and the CA should organize further activities, which should include the participation of a qualified fish health expert. An outbreak investigation requires a systematic approach based on clinical experience in the selection of proper specimens for diagnostic testing and to collect all relevant data about culture conditions, weather conditions, etc. A sufficient number of moribund or clinically affected individuals should be selected for laboratory examination. Apparently healthy fish should also be sampled to find out if there are disease carriers. Dead specimens are not appropriate for laboratory diagnosis. All relevant data concerning the outbreak event should be collected through interviews with staff working with the fish and the farm manager and noted on the form which should accompany the sample.

2.2 Clinical inspection and sampling on a salmonid farm

The culture of rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta m. fario*) and other salmonid species is an important aquaculture activity present to a greater or lesser extent in all Western Balkan countries. Diseases can have negative impact on aquaculture production, and high mortality in salmonid culture is usually associated with disease. Salmonids are mainly cultured in earthen and concrete raceways at the wellsprings, and such farms are typically situated in mountainous regions (Figure 1). The main objective of this section is to give detailed instruction how to prevent the introduction and spread of serious salmonid diseases, and this is done by providing advice and diagnostic services to fish farmers.

Clinical inspections for surveillance of OIE- and EU-listed diseases, as well as for those viral diseases listed in national legislation (i.e. National Pathogen List), namely viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN) and infectious pancreatic necrosis (IPN), are best carried out during a period of the year when the water temperature is below 14°C. When farms are to be subjected to health inspection and/or sampled more than once per year, the interval between the two inspections and/or collection of samples should be as far apart as possible, with not less than four months between inspections or samplings. During the clinical inspection of the farm, all production units (ponds, tanks, net-cages, etc.) must be inspected for the presence of dead, weak or abnormally behaving fish (Figure 2). Particular attention must be paid to the water inlet and outlet areas, as sick fish will tend to congregate in these places (Figure 3). If such fish are not present, rainbow trout or other freshwater salmonids such as brook trout (*Salvelinus fontinalis*) or brown trout that appear normal should be selected for sampling. If rainbow trout are not present in the facility, the sample must to be representative of all other susceptible species present. If more than one water source is utilized for fish production, fish representing all water sources should be included in the sample (Figure 4). The fish selected shall include fish collected in such a way that all parts of the farm as well as all year classes and all species are proportionally represented in the sample. The same principles of clinical inspection and collection of samples should be followed even when where it is suspected that higher mortalities are not caused by OIE- or EU-listed disease agents.

According to EU legislation (*Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals;* see Anon., 2006) adopted by all member states and candidate countries, the sampling schedule and number of specimens in the sample depends on the two or four-year programme carried out to achieve disease-free status. If a two-year surveillance programme is implemented, two samples consisting of 150 specimens per year should be submitted to the laboratory for examination. If a longer surveillance programme is ongoing, a single sample consisting of 30 specimens should be examined for the presence of listed diseases.

Figure 1. Salmonid farm in a mountainous region

Figure 2. Abnormally behaving fish. *(*Photo courtesy of Prof Dr Vlasta Jenčić*)*

Figure 3. Water inlet area where sick fish tend to accumulate

Figure 4. Fish from each water source should be included in the sample. *(*Photo courtesy of Dr Svjetlana Batinić*)*

2.3 Clinical inspection and sampling of a cyprinid farm

Cyprinid culture is the most common form of fish farming practiced in the Western Balkans countries. Different warmwater species such as common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), bighead carp (*Hypophthalmichthys nobilis*) and silver carp (*H. molitrix*), are cultured in extensive or semi-intensive farms (Figure 5). In some cases, non-cyprinids such as wels catfish (*Silurus glanis*), northern pike (*Esox lucius*) and pike perch (*Sander lucioperca*) may also be cultured. The purpose of sampling is important in determining the methods of sample selection, i.e. whether the samples are being taken for routine monitoring or for investigating the cause of a clinical disease outbreak. Prior to sampling (Figure 7), an assessment of the site and husbandry conditions is needed, as well as an inspection of the fish present. For monitoring of disease in aquaculture facilities, sampling is generally conducted to a statistical standard which provides for a 95 percent confidence level of detecting a 2 percent prevalence of disease. With "wild" populations, which are at lower population densities and where sample size can be limited, sampling to give a 95 percent confidence level of detecting disease at the 10 percent level is acceptable. Where overt clinical disease is manifested (Figure 8), sampling will normally be limited to 5–10 affected animals (Figure 6).

2.3.1. Procedures for surveillance of spring viraemia of carp (SVC)

Outbreaks of SVC in carp generally occur at between 11°C and 18°C, rarely below 10°C, and mortalities decline as the temperature exceeds 22°C. Secondary and concomitant bacterial and/or parasitic infections can affect the mortality rate and display of clinical signs. In carp, the disease is often observed in springtime, particularly after cold winters. The poor condition of the over-wintered fish may be a contributory factor in disease occurrence. The disease can occur in fish held in quarantine following the stress of transportation, even though there has been no prior evidence of virus in the fish.

Clinical examination of cyprinid fry and fingerlings (0+ and 1+ age categories) must be carried out in the spring and autumn when the water temperatures are below 18°C. At the same time, samples shall be delivered for testing for the presence of SVC virus.

Examination for the presence of SVC virus in cyprinid broodstock has to be carried out during the spawning time. For the purpose of examination, a sample of ovarian fluid must be taken at the end of squeezing (one pooled sample should include ovarian fluid from five broodstock fish in one tube). The main characteristics of SVC are given in Table 1.

2.3.2. Procedures for surveillance of koi herpesvirus (KHV)

Koi herpesvirus may cause disease in common and koi carps, and it is optimal to sample fish that have been kept for a prolonged time period at the virus permissive temperature range (2–3 weeks at 20°C to 26°C). There are indications that certain management practices (e.g. netting and/or transport of the fish) can reactivate the virus in fish with a carrier status, thus increasing the chance of KHV detection. If it is possible to collect samples 24 hours after such management practices, the chance of KHV detection could be enhanced. For surveillance purposes, pooling of samples is not recommended. However, if pooling is necessary, it is acceptable to pool tissue material from two fish. The main characteristics of KHVD are given in Table 1.

Table 1. Characteristics of koi herpesvirus (KHV) and spring viraemia of carp (SVC) disease.

	Koi herpesvirus (KHV; CyHV-3)	**Spring Viraemia of Carp (SVC)**
Species Affected	Common and koi carp; other species may carry virus	Common carp, koi; goldfish, grass carp, bighead carp, silver carp and Crucian carp
Optimal Water Temperature	18–27°C	5–18°C
Transmission	Direct contact, faecal material, infected water/mud, equipment, vectors	
Age Susceptibility	Young more susceptible than mature fish	
Clinical Signs – Behavioural	Lethargy, swimming close to the surface, respiratory distress, erratic behaviour, uncoordinated swimming	Lethargy, low on tank or pond bottom, awkward swimming
Clinical Signs – External	Gill necrosis, sunken eyes, notched nose, pale patches on the skin, secondary bacterial and parasitic infections	Exophthalmia, petechial skin haemorrhage, abdominal distention, mucus from vent
Clinical Signs – Internal	Few, variable signs but may include greater than normal adhesions in the body cavity and enlargement and/or mottled appearance of internal organs	Oedema, inflammation, pinpoint haemorrhages of many organs including swimbladder

Figure 5. Warmwater pond

Figure 6. During fishing out it is easy to notice clinically diseased carp

Figure 7. Sampling for routine health control

Figure 8. Sick fish swimming close to pond edge

2.4 Clinical inspection and sampling on marine fish farms

The south-western area of the Western Balkan Region forms part of the Mediterranean basin, and thus Bosnia and Herzegovina, the Republic of Croatia and Montenegro all have territorial sea with the mariculture of Mediterranean species, namely European seabass and gilthead seabream. These species are cultured in floating cages of various shapes and sizes, ranging from rectangular (Figure 9) to circular (Figure 10). Regardless of the size or shape, it is always possible to approach to the cage, to walk around it and to observe the appearance of the fish inside the culture unit (Figure 11). Special attention should be paid to any fish swimming separately of the shoal, staying close to the surface (Figure 12) or to the netting, or that are reluctant to eat, are abnormally pigmented (dark or pale) or that are displaying abnormal swimming behaviour. Sometimes diseased fish are not visible from the sea surface; therefore, it is necessary to get information from scuba divers. For instance, outbreaks of pseudotuberculosis caused by *Photobacterium damselae* subsp. *piscicida* usually start with dead fish visible only on the bottom of the cage net.

During the inspection of the marine farm, it is necessary to collect information about the environmental conditions (e.g. temperature, oxygen saturation, weather conditions), culture conditions (movement of fish, stocking density, hygienic and preventive measures employed, recent treatments, handling procedures, feeding procedures) and to observe all age categories (fry, yearlings and market-size fish) and cultivated species. Individuals showing abnormal appearance or behaviour should be collected for diagnostic purposes, and clinically healthy fish should also be sent to the laboratory. All collected data should be recorded on the sample submission form which should accompany the sample. When an outbreak of disease occurs, it is necessary to collect at least ten moribund fish and ten "healthy fish" for laboratory diagnostic procedures.

There are no EU- or OIE-listed diseases for Mediterranean fish species for the moment, but viral nervous necrosis (VNN) is considered to be a threat to the successful farming of marine finfish throughout the Mediterranean area. According to Article 50 of Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals (see Anon. 2006), there is a possibility for member states to lay down specific requirements for surveillance, sampling and diagnostic methods to obtain disease-free status. This is supported by Commission Decision (2010/221/EU) approving national measures for limiting the impact of certain diseases in aquaculture animals and wild aquatic animals in accordance with Article 43 of Council Directive 2006/88/EC (see Anon., 2010).

Bearing this possibility in mind, fish health experts should be ready to perform sampling on marine fish farms and to recognize suspicion of those diseases which could have great economic impact to the national mariculture industry, regardless of their viral, bacterial or parasitic origin.

Figure 9. Rectangular marine cage (8 x 6 x 8 m) at a small farm

Figure 10. Huge, circular sea cage (38 m diameter) at an industrial marine farm

Figure 11. Normal appearance of the shoal inside the cage when viewed from the sea surface

Figure 12. Sick fish swimming close to the water surface

2.5 Clinical inspection and sampling on mollusc farms

Mollusc culture along the eastern Adriatic coast comprises the semi-intensive culture of Mediterranean mussel and European flat oyster, and they have extensive interaction with the natural environment. The culture of both mussels and oysters starts with the collection of naturally spawned spat in production areas (Figure 13). Mussel spat are later introduced into hollow, net-like cylinders which are hung by ropes on rafts (Figure 14) which are spread between holding buoys. Oyster spat is collected in the same way and after some time, they are moved to plastic baskets or bags (Figure 15) that are hung on similar ropes as mussels. In certain areas, fishermen collect natural ("wild") populations using dredges (Figure 16). Those natural populations, albeit often not accessible, are very important to national production and the health status of molluscs, and it is important to include them in surveillance programmes. Molluscs are mobile during some life stages (e.g. oyster larvae), while adult stages are often sessile (e.g. oysters).

All culture installations are submerged at various depths, and it is difficult to inspect an installation without the help of the farmer. Most often, there are no other signs of infection apart from high mortalities. Abnormal mortalities are those mortalities of cultured or natural populations of molluscs which exceed normal mortalities in two weeks or between two inspections. It is necessary to collect information about the pattern of mortalities in the open water and to observe all abnormalities, deformities or shell damage. Environmental conditions (temperature, water quality (e.g. oxygen depletion, presence of algal blooms)) and weather conditions should be recorded.

It is very important to carry out a thorough sampling on a mollusc farm or production area. Sampling should include individuals from the inner and central parts of the production area, as well as from peripheral areas. From all parts of the culture area, specimens should be collected from close to the water surface, from the middle of the water column and from close to the bottom (Figure 17). The number of individuals in the sample depends on the purpose of the sampling. In the case of a clinical outbreak of disease, samples from ten moribund and ten normally appearing individuals should be collected.

If a surveillance programme for the evaluation of health status in the production area is being implemented, the number of the individuals in the sample will depend on whether a two or four-year programme is being implemented. In the case of shorter programme the sample should consist of 150 individuals, while in the case of a longer programme, 30 individuals per sample would be sufficient. To prevent contamination or exhaustion of molluscs intended for disease examination during transportation, it is good to remove mud, dirt and any fouling organisms from the shell before packing (Figure 18).

Figure 13. Collector of naturally spawned oysters and mussels

Figure 14. Mussel culture installation (note net-like cylinders)

Figure 15. Cultivation gear for oysters

Figure 16. Dredging of a natural population of molluscs

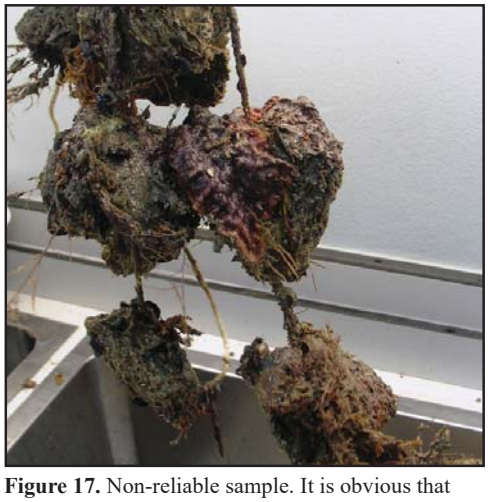

Figure 17. Non-reliable sample. It is obvious that individuals are not collected randomly

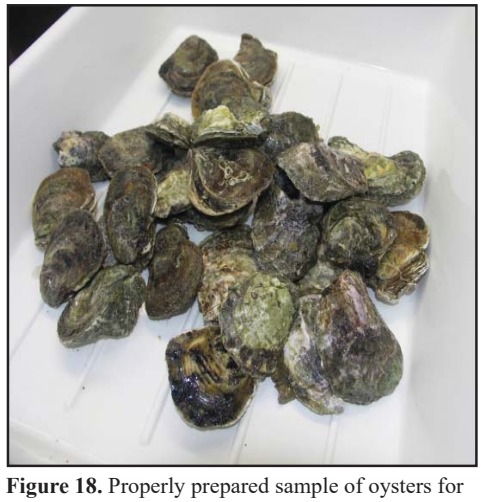

Figure 18. Properly prepared sample of oysters for disease surveillance

2.6 References

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3. Preservation, packing and shipping of samples

3.1 Shipping of live fish

Shipping of live fish together with additional information from the fish farmer regarding the circumstances of the mortalities will provide the fish health specialist with the best possible specimens for analysis. If requested, the fish farmer should also bring water samples from the aquaculture facility. In this manner, the diagnostician will gain useful information regarding the disease. Live fish can be examined for parasites, and pathogenic microorganisms (bacteria and viruses) can be cultured from specimens. Histopathological examination can be performed from appropriately prepared samples taken from organs and tissues that are fixed in different fixatives, allowing the diagnostician to look for tissue changes indicative of a pathological process.

To ship live fish successfully for laboratory examination requires that samples should be packed into a strong, waterproof, insulated shipping container (e.g. a disposable styrofoam cooler). The fish should be placed in a heavy plastic bag filled approximately 1/3 with water from the culture facility and subsequently inflated with oxygen. The bag should be sealed by twisting the opening and securing it closed with several heavy-duty rubber bands or plastic tie-downs; an air-tight seal is essential. An abundant amount of crushed ice should be placed in a heavy plastic bag and the bag placed in the shipping container next to the bagged fish to lower the temperature. A clean plastic mineral water bottle with a tight screw cap filled partially with water and overlaid with oxygen is a convenient way to transport a small number of fry. For fish up to a size of 10 g, a 10 litre plastic container can be used in a similar way. The maximum transport time for live fish depends on the water temperature and ratio between fish biomass, water volume and oxygen. As a general rule, fish biomass should not exceed 1/3 of the water volume, and the transport time should not be longer then 12 hours. Records including the name, address and phone number of the shipper and information describing the outbreak and the culture system should be placed in another plastic bag and put into the box. The shipping container should then be sealed and, it should be clearly indicated which end is “up” and that live fish are enclosed (Figure 19).

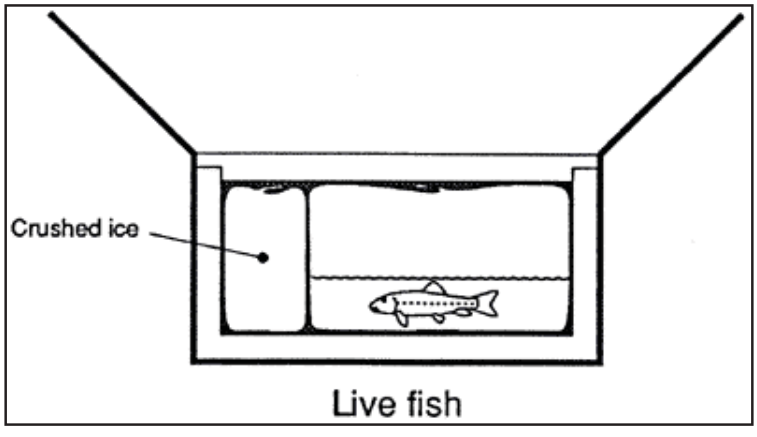

Figure 19. Shipping of live fish

3.2 Shipping of fresh fish

Whole fish, eggs and ovarian fluid may be shipped on ice for laboratory examination in a fresh state. Such samples may have some diagnostic limitations i.e., live parasites may not be present or histopathological examination may not yield reliable information if fish are maintained in an iced condition too long. Iced specimens are usually satisfactory for the culture of bacteria and viruses, but they should be kept at a temperature approaching 0^{o}C without freezing. Samples should be packed in a strong, waterproof container made of insulated material. Each sample should be placed in a plastic bag to avoid spoilage and the bagged samples then placed in a larger, strong plastic bag in the shipping container. A sufficient quantity of cooling blocks should be added to the container. Records, including the name, address and phone number of the

shipper and information describing the event or indicating the reason that the sample is being sent to the diagnostic laboratory should be placed in a separate, small plastic bag and placed into the shipping container. Maximum transport time for fresh, cooled material is 24 hours (Figures 20 and 21).

Figure 20. Styrofoam box for transport of live fish

Figure 21. Fish are packed in a plastic bag and frozen plastic bottles or cooling blocks are used to maintain low temperature during transport

3.3 Shipping of molluscs

Molluscs should reach the diagnostic laboratory alive; hence, a proper packing procedure will ensure this. Mussels should be packed tightly in a net to prevent opening of the shells. Oysters should be packed in a box organized in layers. The concave shell should be placed on the bottom of the package, the first layer covered with wet paper, and then another layer of oysters added, also with their concave shells facing downward. By packing in this manner, the oysters are not able to open their shells and so intervalvular liquid will stay inside and there is no possibility to aspirate any substances from the packaging. Both species should be packed in a cooling box with sufficient freezing blocks (not ice cubes, which could melt and create liquid inside the package) to prevent their temperature from exceeding 10°C (Figure 22). A temperature under 10°C will enable the molluscs to stay alive for several days. All data about sea temperature, weather and culture conditions, as well as any other observations, and the sampling points should be recorded. The written form should be packed into a plastic envelope to protect it from wetting and put into the shipment (Figure 23).

Figure 22. Waterproof boxes for shipping mollucs

Figure. 23. A written record is placed in a plastic envelope that is attached to the box cover

3.4 Shipping of organs in transport media

For the diagnosis of viral diseases, sampling should be performed on the farm (Figures 24 and 25), and small portions of selected organs (i.e. anterior kidney, spleen, heart, gills and brain) placed into plastic tubes containing transport medium. According to agreement with the diagnostic laboratory, they will send tubes containing transport media (cell cultivation media with added fetal bovine serum (FBS), antibiotics, fungicides and buffer) a few days prior sampling. The tubes should be wrapped in plastic and sent with a freezing block in a cooling box to keep the temperature below 10°C without freezing. Organs from ten fish can be pooled in one tube, keeping in mind that the volume of transport medium should be twice than of the organs (Figures 26 and 27). The time from sampling to laboratory processing should not exceed 48 hours (Figure 28). A written record should accompany samples (Figure 29).

Where practical difficulties arise (e.g. bad weather conditions, non-working days, laboratory problems) which make it impossible to process the samples within 48 hours after their collection, it is acceptable to freeze the tissue specimens in transport medium at -0°C or below and carry out virological examination within 14 days. The tissue, however, must be frozen and thawed only once before examination. Records must be kept with details on the reason for each freezing of tissue samples (such as storm, cell lines died, etc.).

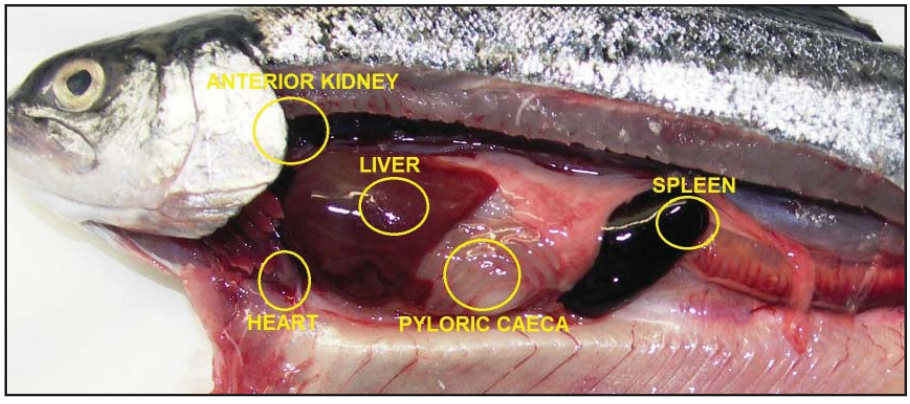

Figure 24. Organs to be sampled for virological examination on a salmonid farm

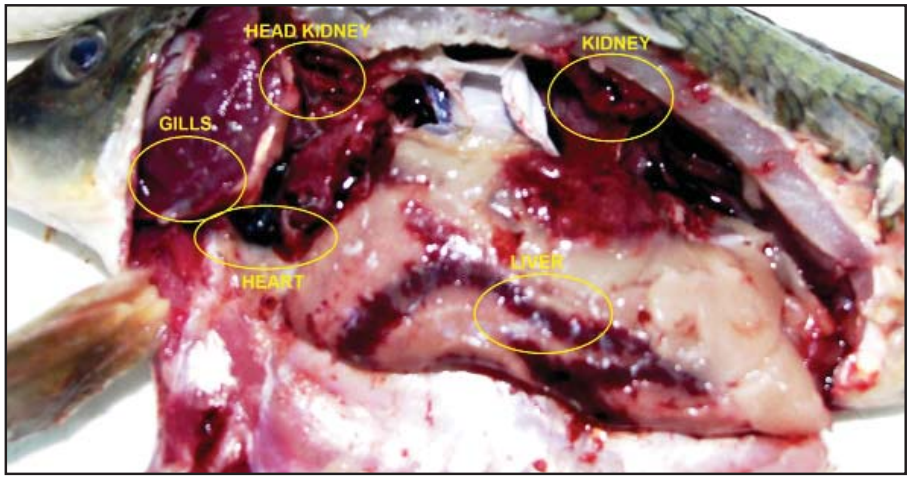

Figure 25. Organs to be sampled for virological examination on a carp farm

Figure 26. Organs from ten fish could be placed into one tube with transport medium

Figure 28. Organs in transport medium in a styrofoam box with freezing block

Figure 27. Volume of sampled organs should not exceed one third of the medium

Figure 29. Written data should accompany the shipment

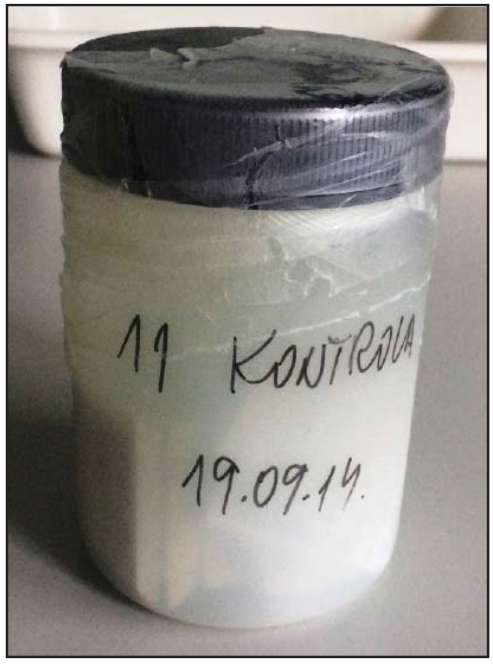

Figure 30. Sealed container containing tissues intended for histological analysis

3.5 Shipping of material for histological examination

Sometimes it is not possible to send whole diseased fish or molluscs to the laboratory for examination. In this situation, organs fixed in the appropriate solution will be sent in plastic bottles or tubes containing fixative. However, it should be kept in mind that the diagnostician must be able to detect tissue changes indicative for the disease process and not of a decomposition process in an animal once death occurs. This can only be done with a carefully processed sample. To safeguard against spillage, the containers should be wrapped in sufficient absorbing paper and placed in sealed plastic bags before placing them in an outer envelope or carton box (Figure 30).

3.6 References

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4. Diagnostic techniques

4.1 *In vivo* examination

Any aberrations in fish behaviour should be taken into account during the clinical inspection of the aquaculture facility. In Table 2 below, the main changes in the behaviour or appearance that can be observed in fish swimming freely in the culture unit are listed.

Table 2. Main possible aberrations in appearance or behaviour of cultured fish.

ABERRATION	POSSIBLE CAUSE
Abnormal skin colouration	Changes of pigmentation: dark colouration is sign of stress due to poor culture conditions or disease; light colouration is usually caused by hypoxia or anaemia caused by parasitic infection or another disease condition
Irregular breathing	Opercular action is increased due to stress caused by hypoxia or gill impairment
Abnormal swimming behaviour	Swimming separately from the lot, close to the surface, sinking to the bottom, loss of balance, cork-screwing or belly-up position are often associated with lethargy and are signs of disease; sometimes fish rub against tank surfaces to dislodge ectoparasites
Reduced feed intake	Reduced feed intake or reluctance to eat is usually associated with stress, but disease should be taken into account as well
Reduced growth rate	Growth in length and weight is measurable and should be in accordance to the expected growth curve. Deviation from the growth curve is a sign of bad welfare, and could indicate a diseased condition
Skin damage	Skin damage could indicate mechanical injuries due to manipulation, predator attacks or the presence of lesions caused by infection (i.e. petechiae, shallow or deep skin lesions, scale loss)
Poor appearance	Including leanness, cataracts, etc. could be a sign of bad environmental conditions, malnutrition or infectious disease

The spread of aberrations from one culture unit to another may indicate an infectious disease, and in such a situation it is necessary to take appropriate measures to prevent further spread of the agent, even when the exact cause of the disease is not yet known.

In some cases, blood samples are collected for diagnostic purposes. It is possible to take blood only from live fish, which can be done immediately after stunning. Anesthesia can also be applied, but in some cases this can interfere with blood properties (e.g. in the quantification of blood proteins), and this should be kept in mind.

4.2 *Post-mortem* examination

Before beginning an external examination, all required equipment should be prepared (Figure 31), including laboratory trays, sterile scissors, forceps and scalpels, glass slides and cover glasses, paper towels, rubber gloves, bottles with saline or phosphate buffered saline (PBS), 95 percent ethanol, fixatives for histology, containers for tissues and fixatives, bacteriological plates for initial cultivation of bacteria, sterile bacteriological loops, alcohol or gas burner, and tubes with transport media if organs for virology will be sampled. If external examination and necropsy will be performed on-site, a cooling box with sufficient freezing blocks will also be required. Finally, vessels with disinfectant where all tissues and other wastes should be disinfected are required. At the end of the post-mortem examination, the work place and all used equipment should be properly disinfected.

4.2.1 External examination

After sampling of fish from the culture facilities for disease diagnosis, the external surface should be examined for any deviations from normal shape, and the skin, eyes, fins and gills should be checked and any changes noted, as well as inspected for the presence of external parasites. The next step is the killing of the sampled individuals by anesthetic overdose or by severing the vertebral column at the line where the head and the body are connected. A thorough examination of skin scrapings and gill tissue using a stereomicroscope should follow. Skin samples are prepared by gently scraping a glass mounting slide along the skin surface from the caudal to the cranial end and then adding a drop of freshwater or saline solution depending on fish species to the glass slide. The gills are examined by cutting a small portion of gill tissue without arch (i.e. the gill filaments), adding a drop of appropriate liquid and covering the preparation with a coverslip. They should be examined by stereomicroscope with 10 and 20x magnification for the presence of skin and gill parasites such as ciliates and gill and skin flukes (Figures 31 and 32). The eyes should be checked for corneal opacity, haemorrhages or digenean parasites (within the vitreous fluid or embedded in the lens). The gills are an organ where it is possible to observe haemorrhages, necrosis, changes in colour, etc. Gill tissue for histological examination should be sampled very soon after euthanasia because tissue decay starts very quickly, especially in the warmer time of the year. Finally, the anus should be checked for protrusion, haemorrhages or any other changes and the presence of abnormal secretion.

4.2.2 Necropsy and internal examination

An individual specimen should be placed on the dissection tray or any other surface with the head on the left and tail on the right side. Approach to the internal organs will be possible after removing the body wall. The first, small incision should be made a little bit in front of the anus to avoid rupture of the intestine. The next cut leads dorsally, to a little above the lateral line, then follows in a cranial direction, until reaching the opercular cavity.The body wall can then be removed, allowing the internal organs to be seen. In healthy fish, there is no free fluid in the body cavity, and the musculature is firm and without the presence of haemorrhages, cysts or necrotic areas. The position of the internal organs may vary somewhat between fish species, but generally, fat tissue is present around the digestive system. The kidney is flat and red and is situated between the spinal cord and the transparent swimbladder. The presence of nodules or haemorrhages in the kidney is indicative of disease. The swimbladder should be checked for the presence of parasites and abnormalities. Sometimes it is possible to find parasites in the body cavity compressing or displacing organs. The normal liver is red, while the spleen is deep red and has sharp edges; roundish edges of the spleen indicates septicaemia. The stomach and intestine may be empty or contain feed; swelling or the presence of blood, liquid or mucous is a clinic sign of disease. The digestive organs should be checked for the presence of worms or other parasites. The appearance of the gonads depends on the age of the fish, its sex, and the time of year. The heart, including the bulbous arteriosus should be distinct and shiny. The swelling of any organ beyond its normal size, the presence of white or grey patches, necrosis or haemorrhages are indications of disease, and should be further investigated to determine the cause (Figures 31 and 32).

4.2.3 Examination of fresh organ preparation

When parasites are suspected in the internal organs, it is necessary to prepare squash preparations of the organs or impression smears, which should be observed by microscope, either as fresh preparations or after appropriate staining (Figures 33 and 34).

Figure 31. Equipment needed for necropsy and sampling

Figure 32. Fish sample prepared to be analyzed

Figure 33. Presence of an isopod ectoparasite (*Ceratothoe oestroides*) in the mouth of European seabass

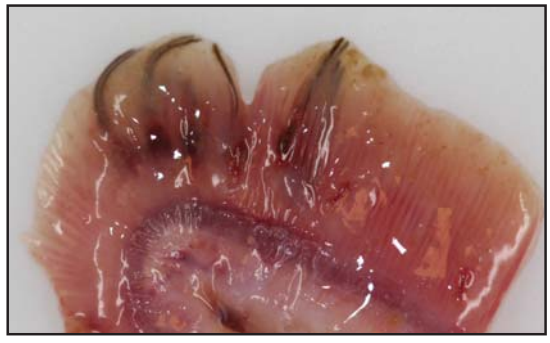

Figure 34. Crustacean ectoparasite (*Lernanthropus kroyeri*) on the gill arch of European seabass (Photo courtesy of Dr Branko Šoštarić)

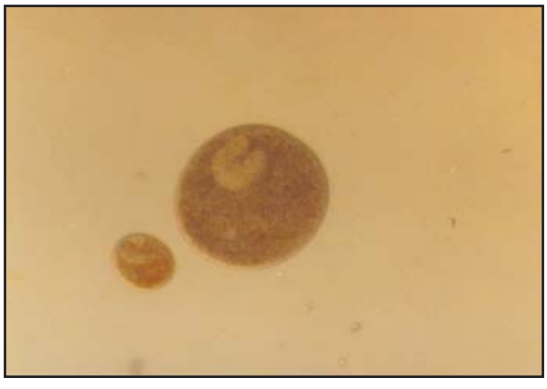

Figure 35. Protozoan infection (*Ichthyophthirius multifiliis*) on carp gills

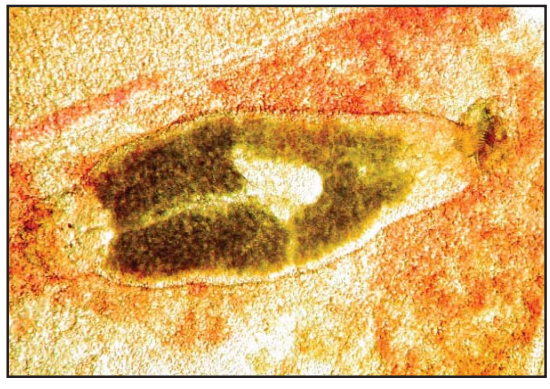

Figure 36. Gill fluke (*Dactylogyrus* sp.)

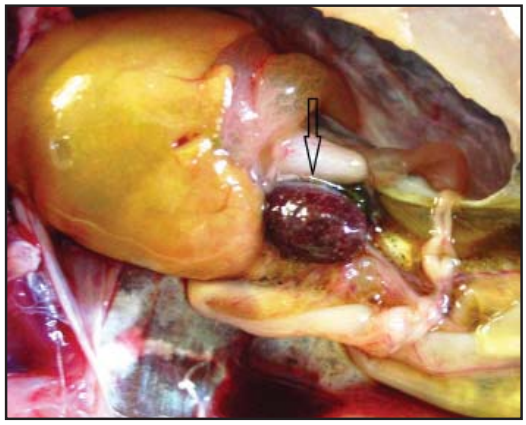

Figure 37. Whitish nodules in enlarged spleen (pasteurellosis caused by *Photobacterium damselae* subsp. *piscicida* in seabream) and steatosis in the liver

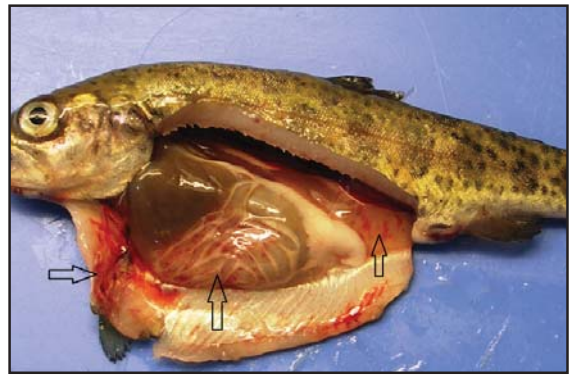

Figure 38. Haemorrhages on the pyloric caeca, peritoneum and intestine (viral hemorrhagic septicaemia in rainbow trout)

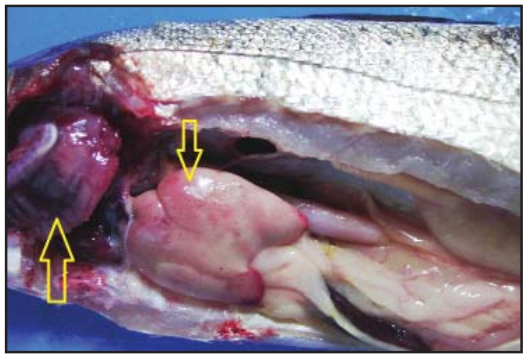

Figure 39. Haemorrhages on the gills and liver indicating bacterial infection (vibriosis caused by *Vibrio anguillarum* in European seabass)

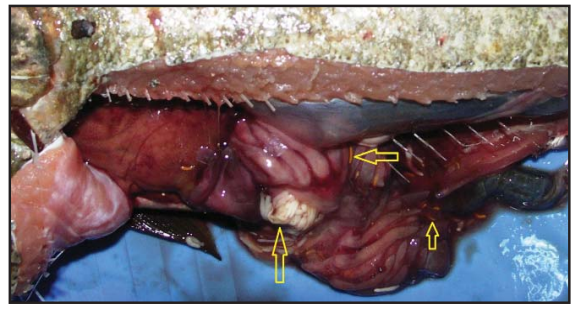

Figure 40. Presence of endoparasites (*Echinorhynchus truttae* and *Cyathocephalus truncatus*) in the intestine of brown trout

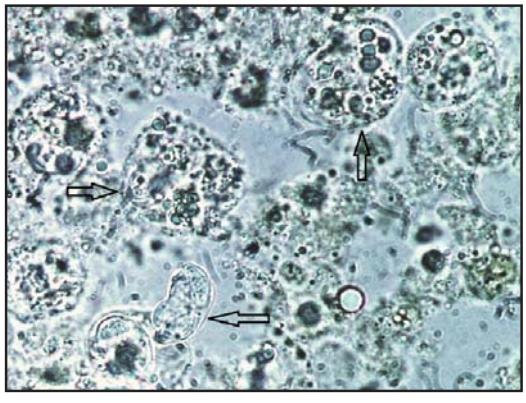

Figure 41. Scraping of seabream intestine infected with pansporoblasts and spores of the myxozoan *Enteromyxum leei* (100x)

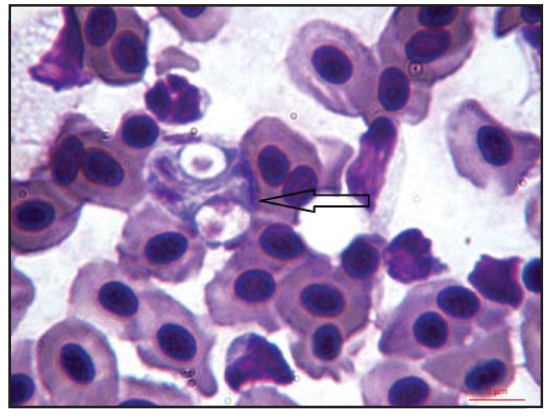

Figure 42. Stage of myxosporean (*Sphaerospora renicola),* the causative agent of swimbladder inflamation of carp (stained by Hemacolour kit) (100x)

4.2.4 Mollusc processing for diagnostics

Molluscs should reach the laboratory alive or fresh, and they should be processed as soon as possible upon arrival. They should be handled in the manner that allows the various sampling techniques for diagnostic purposes to be followed. The technique used to open the shell depends on the species of mollusc, but it is always necessary to cut the adductor muscle to expose the internal body (Figure 43). The soft tissues are situated inside the shell and their appearance should be carefully evaluated. During visual inspection of the soft tissues, the time of the year should be taken into consideration, because both oysters and mussels appear

exhausted after spawning, and this condition should not be confused with a diseased state. The organization of the internal organs of the European flat oyster is shown in Figure 46, while that of the Mediterranean mussel is given in Figure 45. The presence of any visible parasites should be noted. The digestive gland provides favourable tissue for the preparation of smears for the detection of *Marteilia refringens,* while the examination of heart tissue is used to detect *Bonamia*. A transverse section of the soft tissue is taken for histological examination (Figure 44). For molecular confirmation of marteiliosis, a small portion of the digestive gland should be sampled, and for the detection of bonamiosis, a small portion of the gills should be taken.

Figure 43. Opening of mussels

Figure 44. Oysters prepared for sampling of organs for histological examination (the area between the red lines indicates the tissue to be taken)

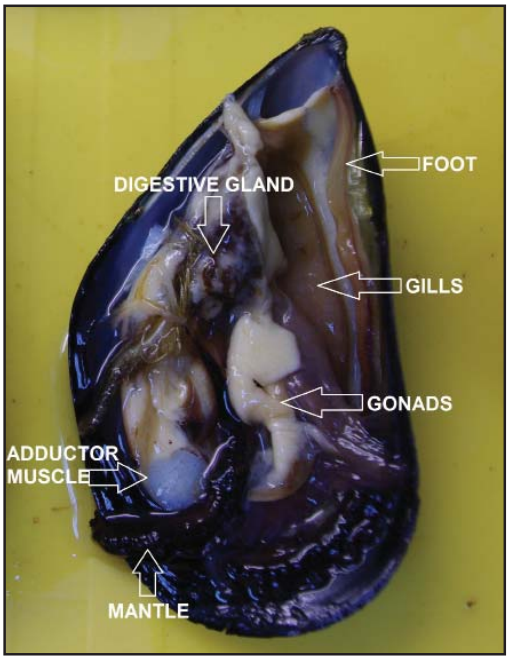

Figure 45. Organization of organs in Mediterranean mussel

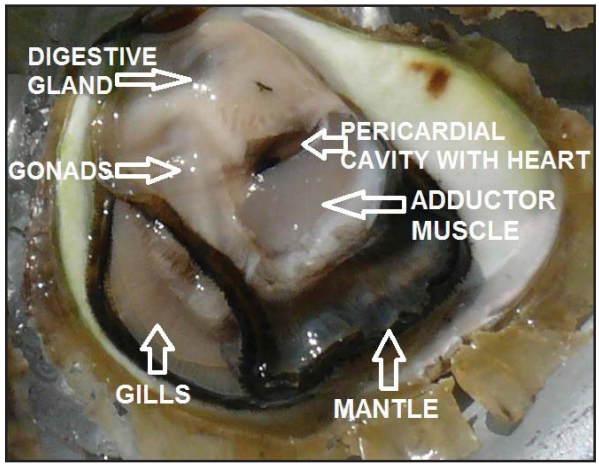

Figure 46. Organization of organs in the European flat oyster

4.3 Bacteriological techniques

4.3.1 Isolation of pathogenic bacteria from finfish

Before necropsy, the body surfaces of the fish, including the gills, tail, fins and eyes should be examined for the presence of lesions and other abnormalities. Samples from lesions can be taken by burning the surface using a hot scalpel, followed by insertion of a sterile loop or swab. Material is immediately plated onto suitable agar medium. After opening the body wall to expose the internal organs (with precaution not to puncture the gastrointestinal tract), the organs should be checked for any visible lesions. Samples for bacteriological examination should be taken following the procedure described above. It is important to take inoculae from the border between normal and abnormal tissue. The anterior kidney, spleen, heart and liver are the most suitable organs for taking samples for bacteriological examination. The majority of fish bacteria will grow on trypticase soy agar (TSA) or blood agar (BA) within seven days. Media such as marine agar (MA) or BA or TSA with the addition of 1.5 percent NaCl can be used for the isolation of bacteria from brackish or marine environments (Figure 47). A number of fish-pathogenic bacteria, such as *Renibacterium salmoninarum*, *Flavobacterium psychrophilum* and *Tenacibaculum maritimum,* need specific growth conditions and thus require especially composed media; they will not grow on the routine bacteriological media.

In all cases, the inoculum is spread using the streak plate technique onto solid medium placed into a Petri dish, providing a large surface for cultivation of micro-organisms. Plates are incubated on 20°C to 24°C. The streak plate technique enables dilution of a sample, and the bacteria present in it will be separated. Sometimes it is necessary to restreak colonies on to fresh medium to achieve pure colonies for identification procedures (Figure 48).

4.3.2 Identification of pathogenic bacteria

Bacterial colonies grown on the agar plate should be checked for morphology, and bacteria examined for motility, staining affinity, biochemical reactions and sometimes, immunological properties. All testing is performed on 24 to 48-hour-old colonies. For presumptive identification, pure bacterial colonies should be Gram stained and initially tested for motility, sensitivity to vibriostat (0/129), oxidase activity, and the oxidation-fermentation (O-F) test applied.

Gram staining of a smear on a glass slide is a basic test for general medical bacteriology (Figure 49). Most fish-pathogenic bacteria are Gram negative. Motility testing determines their capability of independent movement. A single bacterial colony is suspended in a drop of saline, covered by a coverslip and motility is observed using a compound microscope. The movement should not be confused with Brownian movement, which is a random and non-directional vibration exhibited by all bacteria. The oxidase test demonstrates the presence of cytochrome oxydase C, an enzyme situated in the bacterial membrane and involved in the respiratory electron transport chain of bacteria. A commercially available oxidase strip shows a colour change to purple if a cytochrome oxydase C-positive colony is applied (Figure 50). The O-F test is performed to determine if a bacterium is metabolizing glucose and if it does, whether this is done by oxidation (aerobic conditions) or fermentation (anaerobic conditions). Vibriostat testing is carried out using the chemical 0/129, which inhibits the growth of most bacteria belonging to the genus *Vibrio*. All mentioned tests should be performed to achieve a preliminary identification.

Definitive determination of the bacterial species is done using flowcharts detailing biochemical tests. There are several commercially available diagnostic kits such as the BBL™ Crystal™ Identification Systems (the identification method uses fluorogenic and chromogenic reagents and following an incubation period, the sample can be manually read on the Crystal™ panel viewer to obtain a profile number) (Figure 51) or the Analytical Profile Index (API) system, a well-established method for manual microorganism identification to species level that is based on extensive databases. The kits include strips that contain up to 20 miniature biochemical tests (Figure 52).

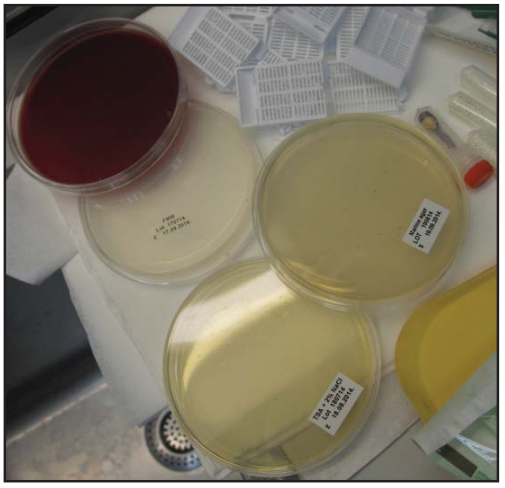

Figure 47. Different bacteriological media for the isolation of fish bacteria

Figure 48. Restreaking of bacterial colonies to achieve pure colony

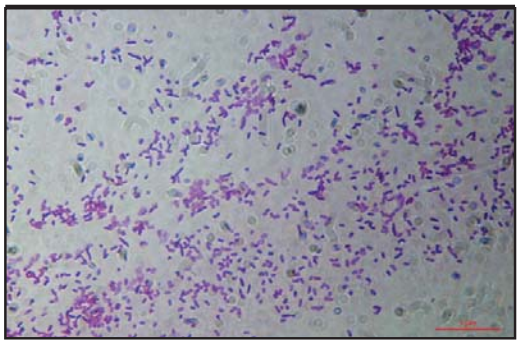

Figure 49. Smear of bacterial colony stained by Gram staining (100x)

Figure 50. Presence of cytochrome oxidase C in isolated bacteria

Figure 51. BBL™ Crystal™ Enteric/ Nonfermenter ID Kit for identification of bacteria

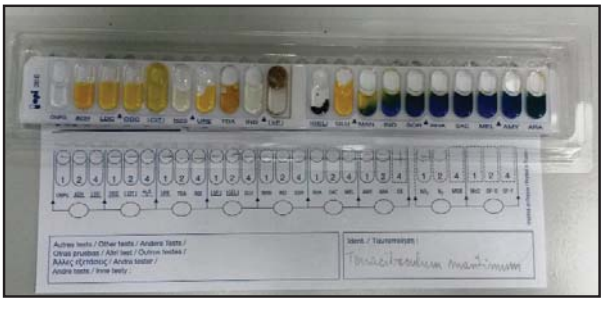

Figure 52. API 20E kit for identification of Gram-negative bacteria

4.3.3 Determination of sensitivity to antimicrobials

Bacterial infections often cause high losses in cultured aquatic animals. In such situations, there is a demand for antimicrobial treatment, usually via antimicrobial substances that are mixed in feed. To minimize possible hazards to aquatic ecosystems and the development of resistant bacterial strains, it is necessary to perform an accurate diagnosis and identify the pathogen, and to chose the most appropriate drug based on the susceptibility testing of the bacterium to antimicrobials. There are two widely accepted methods of sensitivity testing to different antibacterials, namely the disc diffusion method (Kirby-Bauer disc susceptibility test) and determination of the minimum inhibitory concentration (MIC) test. For disc diffusion assay, bacteria are grown on the standard bacteriological plates, harvested and suspended in sterile physiological saline solution adjusted to a 0.5 McFarland turbidity standard. Inoculae are streaked onto Mueller-Hinton agar (MHA) and commercially available discs containing antibacterials are placed onto the agar. The results are read after 24 to 48 hours, and the sensitivity is determined according to the specific diameter zone of inhibition around the disc (Figure 53), as shown in Table 3.

Table 3. Criteria for determination of sensitivity of bacteria to antimicrobials in vitro.

Antimicrobial	Disc content (µg)	Resistant (≤)	Intermediate	Sensitive (≥)
Oxytetracycline	30	8	17	21
Amoxycylin	10	14	14–16	17
Trimethoprim/ sulfamethoxazole	25	10	11–15	16
Florfenicol	30	14	15–18	19
Flumequine	30	16	17–19	20
Erithromycin	15	13	14–17	18
Nalidixic acid	30	15	16–20	21

Determination of MIC better demonstrates the behaviour of the bacterial isolate to antimicrobial molecules. A stock solution of antimicrobial is prepared in appropriate buffer according to the manufacturer's instructions and serially diluted to reach a final concentration of 250 to 0.015 µg ml^{-1}. Bacterial cultures are seeded in appropriate broth at a concentration of 1.5 x 10^8 cells ml^{-1}. The highest dilution of the antibiotic at which no growth is visually determined is considered as the MIC. It is very useful method for determination of developing resistence of bacteria to antimicrobials or when there is a need to study the usefulness of an antimicrobial in the treatment of a bacterial disease (Figure 54).

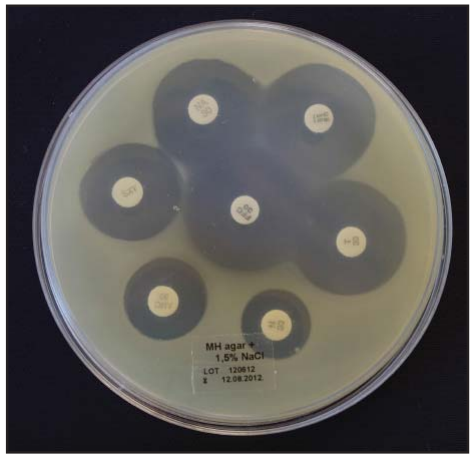

Figure 53. Susceptibility of isolated bacteria to antimicrobials (disc diffusion method)

Figure 54. Susceptibility of isolated bacteria to antimicrobials (minimal inhibitory concentrations)

4.4 Virological techniques

4.4.1 General requirements for the virology laboratory

The laboratory should be organized such that there is no possibility for cross contamination, which means that dissection should be the first step of the process, and that cell culture propagation should be performed in a separate laminar flow apparatus (LFA) with no possible contact with infectious material. All procedures performed with fish viruses should be done in separate facility, i.e. a separate LFA. Laboratory equipment (i.e. pipettes, incubators) must be correctly calibrated, and they should operate within the allowed range. Centrifuges should be calibrated for speed and temperature; spectrophotometers should also be calibrated in proper time intervals, as well as microscopes. All these elements are important to ensure accurate testing results.

4.4.2 Sampling and processing of organs for virological examination on cell cultures

Upon arrival of the sample at the diagnostic laboratory, the accompanying documentation must be checked to register the sample and the temperature inside the package must be measured. If these conditions are satisfactory, it is possible to start with the diagnostic protocols.

Further procedure depends whether whole fish or organs in transport medium have been submitted. If whole fish have been submitted, it is necessary to sample organs depending on the virus for which the sample will be examined (See Sections 2.2 and 2.3).

Organs sampled in the laboratory or those submitted in transport media (Earl's minimum essential medium (EMEM) with 10 percent fetal bovine serum (FBS) and pen/strep buffered with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or TRIS (tris(hydroxymethyl)aminomethane) buffer should be homogenized in a stomacher or mortar and pestle with sterile sand, diluted with transport medium at a ratio of 1:10, centrifuged at 5 000 rpm at 4°C for 20 minutes, filtered through 450 µm sterile filters and incubated for 4 hours at 15°C or overnight at 4°C. The period between sampling and inoculation on cell culture should not exceed 48 hours. If some unpredictable situation occurs, a sample can be frozen at -80°C and the inoculation of cell line can be prolonged for up to 15 days (Figures 55 to 61).

Figure 55. Homogenization of sampled tissue and dilution with "transport medium"

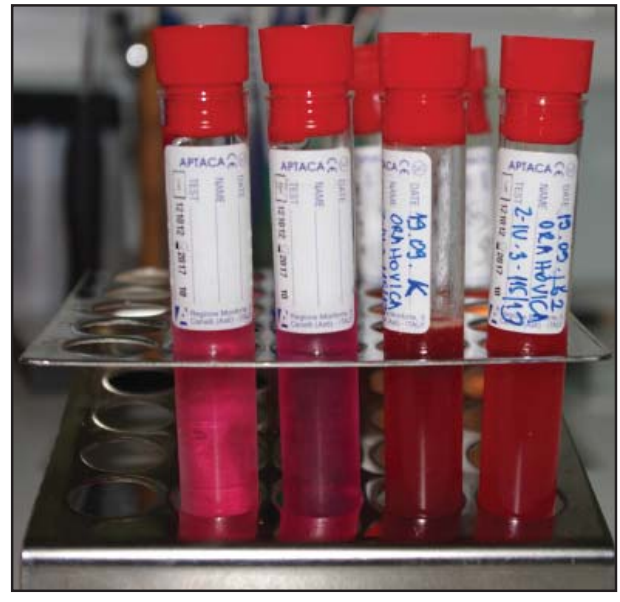

Figure 56. Homogenized and diluted material

Figure 57. Material should be centrifuged in a cooling centrifuge (4°C) for 20 minutes at 5 000 pm

Figure 58. Centrifuged material for virological examination

Figure 59. Filtration through 0.45 µm pore to avoid bacterial contamination

Figure 60. Material ready for virological inoculation on to cell cultures

4.4.3 Preparation of cell cultures for virological examination

According to the EU Commision Decision 2015/1554, virus isolation on cell culture is the "gold standard" for the diagnosis of listed viral diseases. Suggested susceptible permanent cell lines are:

- EPC – *Epithelioma papulosum cyprini* – epithelial cells
- BF – Bluegill fin – fibroblast cells
- FHM – Fathead minnow – epithelial cells
- RTG 2 – Rainbow trout gonads – fibroblast cell

It is compulsory to inoculate material to one epithelial (EPC or FHM) and one fibroblast (BF2 or RTG-2) cell line. Each cell line is growing at 20°C to 30°C in suitable medium (EMEM – Eagle's minimal essential medium with Earle's salt supplemented with 10 percent fetal bovine serum (FBS) and antibiotics). They are propagated and maintained in closed bottles for cell line cultivation. Cell lines for virus isolation are propagated in open systems; 24 or 96 wells plate with flat bottom and open systems should be buffered with the addition of TRIS-HCl or HEPES or incubated in an incubator with CO_2, to maintain pH at 7.6±0.2. Cell cultures used for inoculation of material for virus isolation should be young (4 to 48 hours) and actively growing (Figures 61 and 62).

4.4.4 Virus isolation on the cell cultures

Organ suspensions for inoculation of cell lines should be antibiotic treated or filtered through a filter with a pore size of 450 µm and diluted 1:10 and 1:100 (Figures 63 and 64). Dilutions are prepared on a plate with a round bottom. For each dilution, a minimum of about 2 cm^2 cell area corresponding to a well of a 24 wells plate or 4 wells of a 96 wells plate should be utilized on each cell line. Inoculated cell lines are incubated at 15±2°C for 7 to 10 days; each plate should have some wells of negative control and positive controls (Figures 65 and 66). Cell lines used for virus isolation should be tested for susceptibility to virus every six months. Inoculated cell cultures should be checked by inverted microscope at least three times a week for the presence of cytopathic effect (CPE). If obvious CPE appears, supernatant should be submitted to identification. If there is no CPE during the primary incubation of 7 to 10 days, subcultivation is performed to fresh cell cultures using a cell area similar to that of the primary culture. Aliquots of medium (supernatant) from all cultures/wells constituting the primary culture are pooled and inoculated into homologous cell cultures. If bacterial contamination appears, supernatants should be centrifuged at 5 000 rpm at 2°C – 5°C for 20 minutes, succeeded by filtration. The inoculation may be preceded by preincubation (1 hour at 15°C or 18 hours at 4°C) of the dilutions with the antiserum to IPN virus (equal parts of antisera to different serotype of IPN) to avoid development of CPE caused by this virus. This is very useful in countries with more than 50 percent of farms infected. If no CPE appears during the second 7 days of incubation, the sample should be considered NEGATIVE, while if evidence of CPE appears in cell lines, supernatants should be submitted to identification by neutralization, enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) or polymerase chain reaction (PCR).

Figure 61. Incubator with different cell cultures for virus isolation

Figure 62. All operations should be performed in a sterile laminar flow unit

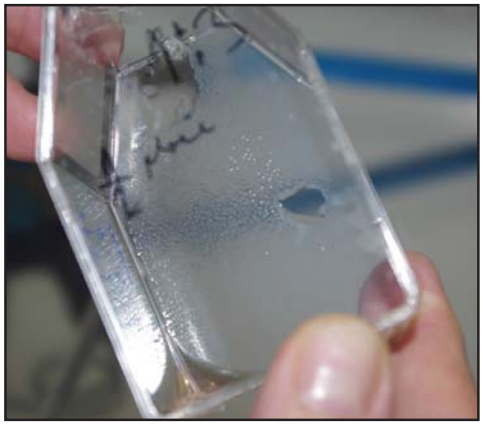

Figure 63. Bottle with trypsinized cell culture

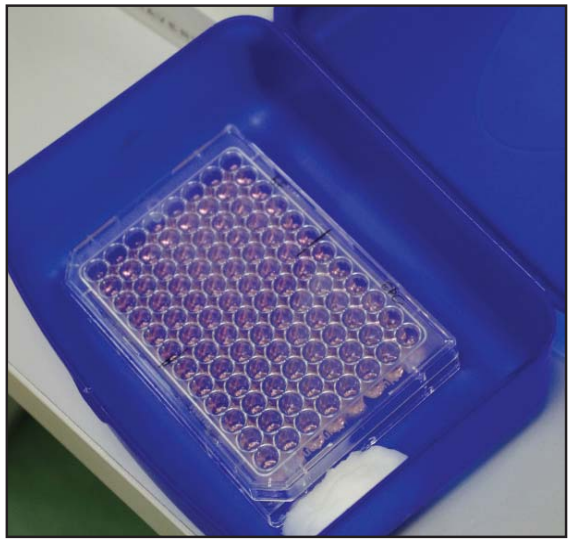

Figure 64. 96-well plate with EPC and BF2 cell lines

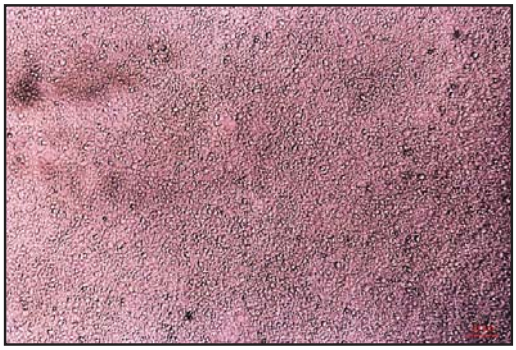

Figure 65. EPC healthy cell line (40x)

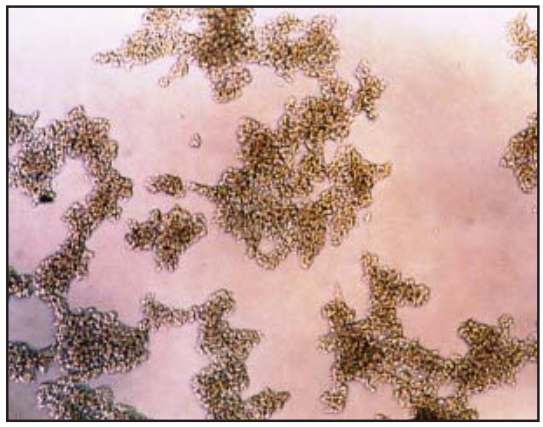

Figure 66. Cytopathic effect caused by virus on EPC cell line (40x)

4.4.5 Immunological methods for virus identification

All these methods are based on the antigen-antibody affinity to detect and visualize the presence of either antigen or antibody in the sample. Well-characterized polyclonal or monoclonal antibodies are essential for immunological methods.

4.4.5.1 Virus neutralization

Supernatant from cells showing CPE should be centrifuged or filtered to remove cells and then diluted. It is then mixed with aliquots of serum containing polyclonal or monoclonal antibodies required to neutralize the homologous virus against which the antibody was raised, and medium alone used as a positive control. Susceptible cells are inoculated and the test is left to incubate until plaques are seen or CPE is complete. The dose of test virus neutralized by the antiserum is compared to that neutralized by the homologous virus. Equivalent neutralization indicates that the test virus is the same as the homologous virus. Development of CPE is checked as has been described previously and the absence of CPE confirms the presence of the particular virus. It should be taken into consideration that some viral strains do not react in neutralization tests, and therefore such isolates should be identified by IFAT or ELISA.

4.4.5.2 ELISA (enzyme-linked immunosorbent assay)

Samples (supernatant) are incubated in wells of microtiter plates coated with dilutions of purified immunoglobulin of antibodies of reference quality which should capture antigen from the sample. The process includes rinsing of the wells and identification of the antigen (virus) by adding biotinylated antibodies of a specificity corresponding to that of the coating antibodies and another period of incubation. After rinsing, horseradish peroxidase (HRP)-conjugated streptavidin is added and allowed to react during another incubation. After last rinse, bound enzyme is visualized using appropriate ELISA substrates (ortho-phenylenediamine (OPD) or others). The intensity of the colour reaction can be read optically by spectrophotometer to provide a qualitative measurement that normally will be proportional with the amount of antigen in the sample (Figures 67 and 68). There are several commercially available ELISA kits for detection of fish viruses in cell supernatant.

Figure 67. Wells in microtiter plates for detection of different viruses

Figure 68. ELISA reader

4.4.5.3 Immunofluorescence technique

Immunofluorescence is the labelling of antigens or antibodies with fluorescent dyes which emit green fluorescence under ultraviolet (UV) light. There are two classes of this technique:

- *Primary or direct immunofluorescence*, where antigen is fixed on the slide and fluorescein-labelled antibodies are layered over it. The slide is washed out to remove unattached antibodies. Examined using a UV microscope, the site where antibody attaches to its specific antigen will show green fluorescence.
- *Secondary of indirect immunofluorescence* is a rapid, specific and sensitive method for detection and identification of viruses in cell culture. After fixation of cells with formalin or acetone-ethanol, primary unlabelled antivirus antibodies are added, followed by FITC (fluorescein isothiocyanate)-labelled secondary antibodies. Results are evaluated using a UV inverted microscope and the sites where FITC-labelled antibody attaches to antigen will show green fluorescence.

4.5 Histology

Histology plays a central role in fish disease diagnosis. It encompasses the scientific area concerned with the structure of tissues. Histopathology provides information on the processes and changes in the tissues, the appearance of organ structure or cell types, and findings that may indicate the cause of disease. Different staining techniques reveal the presence of parasites, bacteria or fungi, as well as lesions in tissues caused by their propagation. Histology is the "gold standard" method for the diagnosis of the listed molluscan diseases marteiliosis and bonamiosis.

4.5.1 Tissue fixation and processing for histological purposes

Proper sampling of tissues for histology is a vital part in the diagnostic procedure because fixation retards alteration of tissue structure subsequent to death and maintains its normal architecture:

- It is necessary to select tissues with lesions typical for the disease condition; moreover, to include the border between "normal" and changed tissues.
- All relevant organs should be sampled for histological examination.
- Tissues should be collected within a few minutes after stunning the fish to avoid autolysis.
- Pieces of tissues for fixation should not be bigger than a sugar cube, and the amount of fixative should be at least ten times greater than amount of tissue.

Although there are several different fixatives, the most often used is 10 percent buffered neutral formalin, which exceptionally for marine organisms, can be replaced with the addition of filtered seawater to a 37 percent formaldehyde solution. Sometimes it is recommended to use Bouin's fluid, which gives better nuclear staining. For fixation of marine molluscs, two fixatives are used; Davidson's fluid for the preparation of histological slides and Carson's fixative, which is appropriate for studying ultrastructure. Sampled tissues are placed into plastic cassettes (Figure 69) that are immersed in the appropriate fixative for at least 24 hours

(Figure 70). Once samples are fixed, they must be dehydrated through an increasing concentration of ethanol (Figure 71) because tissue contains water, and alcohol is used to remove it. The tissue is then treated with xylene to become transparent. The next step is infiltration of the tissue by paraffin and embedding in it. For light microscopy, tissues should be placed in the paraffin to form a block (Figure 72). The plastic cassette should be removed, then the paraffin block is cooled, trimmed and sectioned to 2µm to 5µm thickness using a rotary microtome, and the sections placed on precooled glass slides (Figure 73). The paraffin must first be removed from the section, after which the tissue is rehydrated and stained with specific dyes which permit differentiation of specific cellular components. After staining, the section is again dehydrated so that the coverslip may be permanently affixed by the use of a suitable mounting medium. The coverslip not only protects the tissue from damage but also is necessary for viewing the section with the microscope (Figure 74).

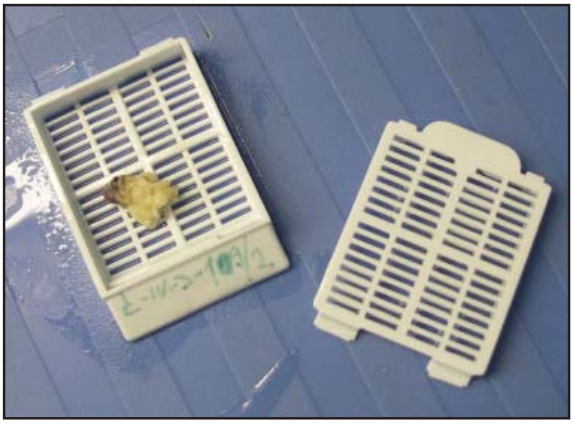

Figure 69. Tissue placed into the plastic cassette

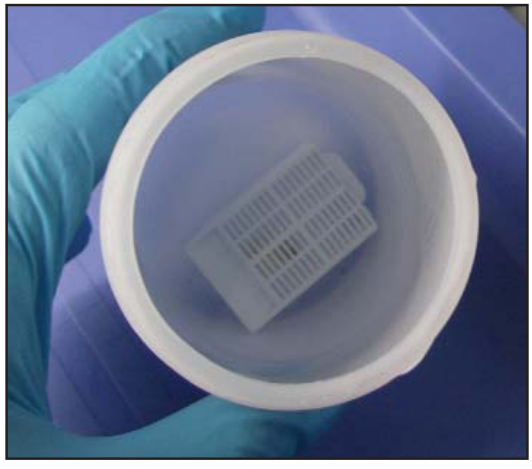

Figure 70. Cassettes with tissues are fixed in formalin for at least 24 hours

Figure 71. Dehydration of tissues in a tissue processor

Figure 72. Embedding of processed tissue in paraffin

Figure 73. Tissues embedded in paraffin blocks, ready to be cut

Figure 74. Histo-slide with hematoxylin-eosin (H&E) stained tissue mounted with a coverslip

4.5.2 Staining techniques

Different staining techniques allow visual differentiation between various microstructures and recognition of normal and abnormal tissue structures. The most commonly used staining technique is hematoxlyn and eosin (H&E) (Figure 75), where hematoxylin is a base that preferentially colours the acidic components of the cell with a bluish tint. The most acidic components are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA); thus the nucleus and region of the cytoplasm rich in ribosomes stain dark blue (referred to as basophilic). Eosin is an acid that dyes the basic components of the cell a pinkish colour. Cytoplasmic components of the cell have basic pH; thus they stain pink and are referred to acidophilic. Masson trichrome stain (Figure 77) is used for connective tissues, and the collagenous components are stained a green or blue colour and the cytoplasm is varying shades of red. Toluidine blue (Figure 76) is a stain that shows general cell structure in the same way as H&E does and thus provides only minimal information about the chemical makeup of a tissue or organ. It is also used clinically due to its strong affinity for the granules in mast cells, one of the wandering cells of connective tissue, and therefore, this staining is often requested as a specific stain for mast cell tumours.

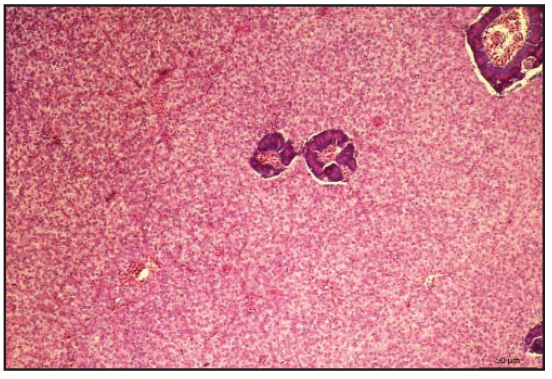

Figure 75. Photomicrograph of carp liver tissue with several islands of pancreatic tissue (hematoxylin and eosin staining) (20x)

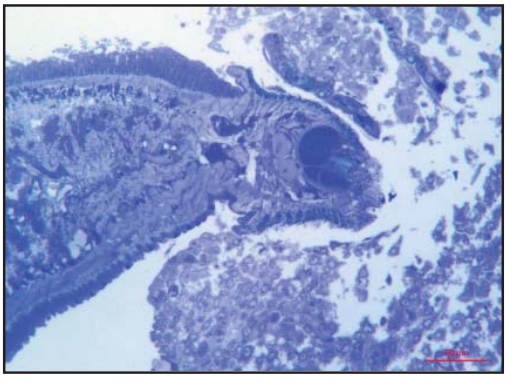

Figure 76. Opisthaptor of the monogenean *Diplectanum aquans* embedded in desquamated gill epithelium of the European seabass (Toulidine blue, 20x) (Photo courtesy of Prof Dr Ivona Mladineo)

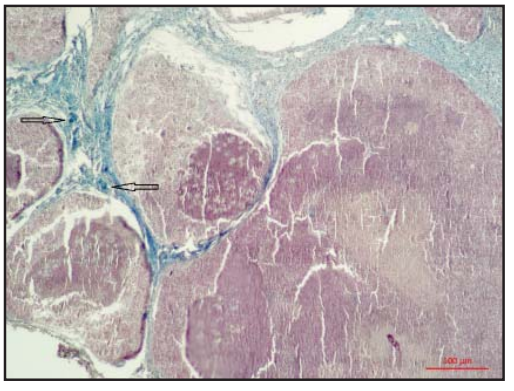

Figure 77. Xenoma-forming *Microsporidium* sp. in tissue of *Knipowitschia croatica* (Masson's trichrome 40x) (Photo courtesy of Prof Dr Ivona Mladineo)

4.6 Molecular methods

4.6.1 Polymerase chain reaction (PCR)

This is a revolutionary method developed by Kary Mullis in the 1980s based on the ability of DNA polymerase to synthesize a new strand of DNA complementary to the offered template strand. Nucleic acid amplification is a valuable tool for diagnosis of aquatic animal diseases. Several amplification methods are

available: PCR (which can be performed as a single or nested assay), reverse transcription PCR (RT-PCR), multiplex PCR and quantitative or real time PCR. (Figure 81)

PCR is used to amplify certain regions of DNA (Figure 78). Short oligonucleotide primers are designed that will hybridize to each end of the target region of DNA to be amplified, on opposite strands of the DNA. The reaction includes:

- template DNA that may be in various forms, from a simple tissue lysate to purified DNA primers
- polymerase enzyme to catalyze creation of new copies of DNA complementary to the target sequence
- nucleotides to form the new copies

During each round of the thermo-cycling reaction, the template DNA is denatured (Figure 79), primers anneal to their complementary regions and polymerase enzyme catalyses the addition of nucleotides to the end of each primer, thus creating new copies of the target region in each round. Theoretically, the increase in amount of product after each round will be geometric. The presence or absence of a product following PCR may be sufficient to indicate whether a sample is infected by a certain pathogen. This is done in an automated cycler (a thermo-cycler) which can heat and cool the tubes with the reaction mixture in a very short time.

Agarose electrophoresis is performed to visualize the PCR products. This step allows determination of whether the PCR was successful, the resulting product is the correct size, whether other products were amplified as well, and whether primers were bonded to the correct site. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode and away from the negative electrode. Several factors influence how fast the DNA moves, the most important being the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. The DNA is visualized in the agarose gel by the use of a dye binding to DNA (Figure 80).

In some situations, the specificity of diagnosis can be improved by further manipulations of PCR products, and the amplification is only carried out in order to enable these other reactions. *In situ* hybridization or nucleotide sequencing using PCR products can often provide further detail on the identity of the original sample material.

4.6.2 Gene sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called sequencing**.** It has become a powerful technique, allowing analysis of genes at the nucleotide level. In the automated fluorescent sequencing, fluorescent dye labels are incorporated into DNA extension products using 5-dye labeled primers (dye primers) or 3-dye labeled dideoxynucleotide triphosphates (dye terminators). A DNA sequencer detects fluorescence from four different dyes that are used to identify the A, C, G and T extension reactions. Each dye emits light at a different wavelength when excited by an argon ion laser. All four colours and therefore all four bases can be detected and distinguished in a single gel lane or capillary injection. Cycle sequencing is a method in which successive rounds of denaturation, annealing and extension in a thermo-cycler result in linear amplification of extension products. The products are then loaded onto a gel or injected into a capillary. DNA Sequencing Analysis Software analyzes the raw data and calls the bases. Analysis software analyzes raw data to quantify the DNA fragments and determine the size of the fragments by comparing them to fragments contained in a size standard. The sample tubes are placed in a tray in the instrument's autosampler. The autosampler successively brings each sample into contact with the cathode electrode and one end of a glass capillary filled with polymer. An anode electrode at the other end of the capillary is immersed in buffer. A portion of the sample enters the capillary as current flows from the cathode to the anode. This is called electro-kinetic injection. The end of the capillary near the cathode is then placed in buffer. Current is applied again to continue electrophoresis. When the DNA fragments reach a detector window in the capillary coating, a laser excites the fluorescent dye labels. Emitted fluorescence from the dyes is collected by a charge-coupled device (CCD) camera. The software interprets the result, calculating the size or quantity of the fragments from the fluorescence intensity at each data point (Figure 82).

4.6.3 In situ hybridization

In situ hybridization (ISH) is a method of localizing and detecting specific mRNA sequences in morphologically preserved tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest. Normal hybridization requires the isolation of DNA or RNA, separating it on a gel, blotting it onto nitrocellulose and probing it with a complementary sequence. The basic principles for in situ hybridization are the same, except one is utilizing the probe to detect specific nucleotide sequences within cells and tissues (Figure 83). The sensitivity of the technique is such that threshold levels of detection are in the region of 10–20 copies of mRNA per cell.

Figure 78. DNA extraction from tissue or pure bacterial or viral culture

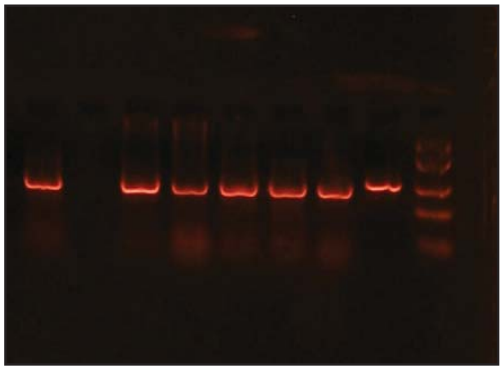

Figure 80. Visualization of DNA in agarose gel

Figure 79. Thermo-cycler

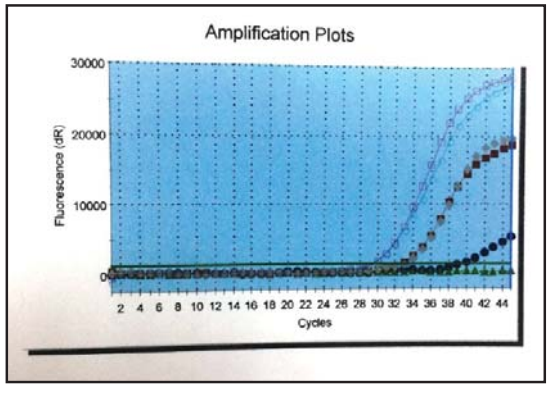

Figure 81. Curve showing cycle threshold (ct) during RT-qPCR

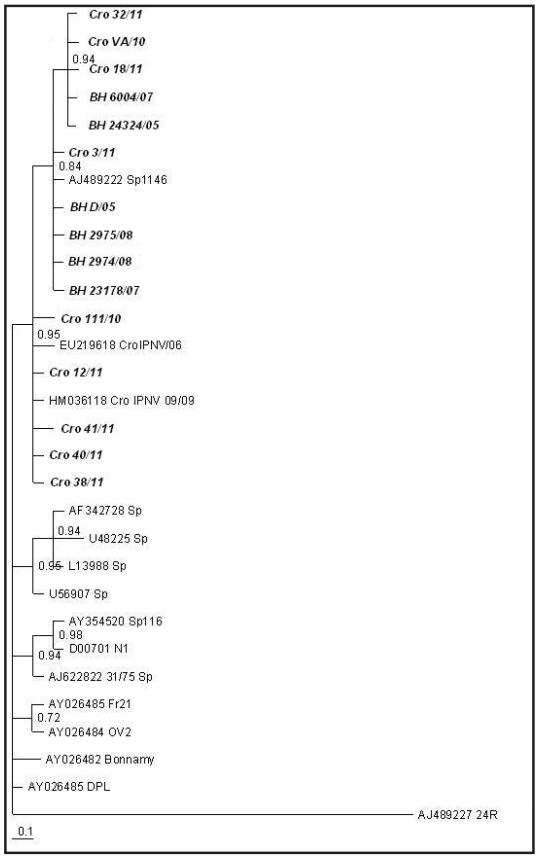

Figure 82. Sequencing as a substrate for phylogenetic and epidemiological research

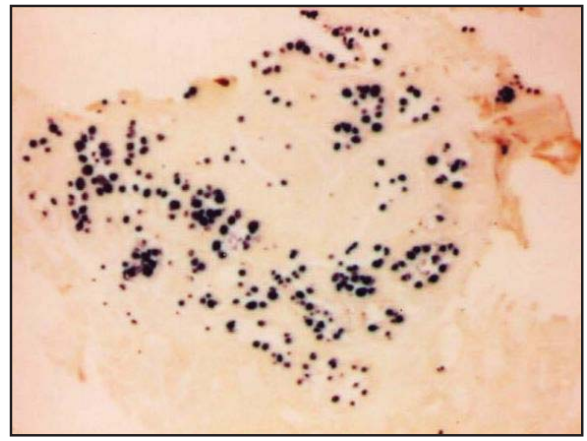

Figure 83. *In situ* hybridization visualizes much more pathogen compared to histology (*Martiela refringens* in the digestive gland of mussel, 40x)

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5. Pathogens of regional importance

5.1 Viral diseases

The rapid growth of aquaculture and the trend towards intensification of fish-farming systems increase the complexity of the health challenges for the industry. Viral diseases are one of the most important threats to the productivity of fish farms worldwide. Unlike viruses of terrestrial animals cultured at uniform temperatures, fish viruses are more specific in terms of temperature tolerance. Variation in temperature range may often induce latency. Many viral diseases of fish are species and geographically limited; fish farms in disease-free areas are considering them as exotic diseases, and they require certification of fish introduced from suspected areas. Generally, viral diseases produce high mortality in young fish and lower mortalities in adults, which may become carriers. Therefore, it is necessary to avoid importation of carriers as well. Specific testing procedures are applied for detection of affected fish and carriers.

There are no effective drugs to treat fish viral diseases. Recently, some vaccines to control certain viral diseases have been introduced. During the outbreak of a viral disease, is possible to manage it by minimizing stress and crowding, employing biosecurity measures and endeavouring to enforce innate immunological mechanisms. The OIE sets out standards and guidance for the improvement of aquatic animal health and welfare of farmed fish worldwide, including standards for safe international trade through the *Aquatic Animal Health Code*. The European Commission and national competent authorities should implement the health measures in the OIE Code to provide early detection, reporting and control of pathogens of aquatic animals to prevent their transfer via international trade while avoiding unjustified sanitary barriers to trade.

The OIE Code provides criteria for listing the diseases of importance for the aquaculture industry. Thus, the viral diseases of finfishes that listed by the OIE are epizootic haematopoietic necrosis (EHN), infectious salmon anaemia (ISA), infection with salmonid alphavirus (SAV), infectious haematopoietic necrosis (IHN), red seabream iridoviral disease (RSID), koi herpesvirus disease (KHVD), spring viraemia of carp (SVC) and viral haemorrhagic septicaemia (VHS). According to EU legislation, EHN is listed as an exotic notifiable disease, while VHS, IHN, KHV and ISA are non-exotic notifiable diseases. National legislation includes viral diseases from these two lists. In this section, we have decided to include EU-listed diseases (VHS, IHN, KHV) with the exception of ISA, which is not of regional importance, as well as certain viral diseases with economic impact to the regional aquaculture industry (SVC, IPN, VER).

5.2 Viral haemorrhagic septicaemia (VHS)

Viral haemorrhagic septicaemia is one of the most important and devastating infectious diseases of rainbow trout (*Oncorhynchus mykiss*), but there were also outbreaks in brown trout, grayling (*Thymallus thymallus*), northern pike and turbot (*Scophthalmus maximus*). The disease is caused by an enveloped RNA virus belonging to the Family Rhabdoviridae, genus *Novirhabdovirus*. The virus is serologically homogeneous but there are four genotypes (I to IV) determined according to the N and G gene sequences, and their appearance is associated with the geographic distribution. Genotype I with its sublineages contains freshwater European isolates and marine isolates from the Baltic Sea and other northern European areas; genotype II contains Baltic isolates; genotype III contains isolates from the North Atlantic area, Norwegian coast; while genotype IV contains North American, Japanese and Korean isolates. The virus survives for a long time outside the fish at temperatures under 20°C; 28 to 35 days in freshwater at 4°C; but in marine water, it is inactivated within 4 days at 15°C. The virus is sensitive to a number of common disinfectants.

VHS is a septicaemic disease affecting the mentioned species, but the virus also replicates in different salmonid and nonsalmonid species. The incubation period under farm conditions is from one to three weeks, depending on fish age, environmental conditions and virus concentration, and sometimes may be up to three months. The most severe outbreaks occur mainly in spring, at water temperatures between 10 and 14°C, with high mortalities during the acute course. Affected fish are lethargic, dark pigmented, show exophthalmia, and have haemorrhages in the eyes and at the fin bases (Figure 84). Petechial haemorrhages are usually

present on the gills, on the skin, and in the musculature, visceral fat, heart, liver, swimbladder and intestine (Figures 85 and 86). Ascites is a usual clinical sign. Disease in fry is characterized by petechial haemorrhages in the yolk sac and distended intestines. Dark pigmentation, pronounced exophthalmia, anaemia and pale liver with haemorrhages are the main clinical signs of the chronic course. Sometimes it is possible to observe swimming disorders associated with low mortality rate due to neural syndrome. Preliminary diagnosis is based on the epidemiological data, clinical appearance of the disease and the absence of bacterial septicaemic infections. Attention should be paid to differentiate VHS from other bacterial or viral septicaemic diseases.

The "gold standard" for diagnosis of VHS is isolation of the virus on cell culture (EPC, FHM, BF-2 or RTG-2) and subsequent identification using ELISA test, IFAT or VN (virus neutralization). Molecular methods like RT-PCR or real-time PCR (RT-qPCR) and gene sequencing are very suitable for virus identification and typing (Figure 89).

Figure 84. Haemorrhages on the fin bases and in the eyes are typical clinical signs

Figure 85. Uni- or bilateral exophthalmia is often visible in infected fish

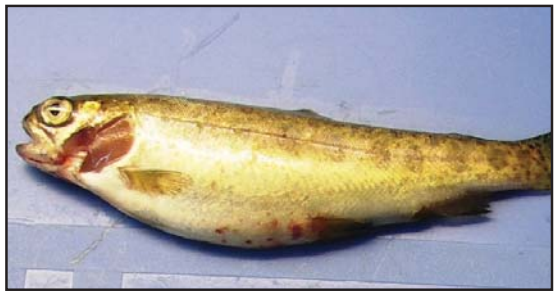

Figure 86. Distended abdominal wall due to ascites, and haemorrhages on the skin

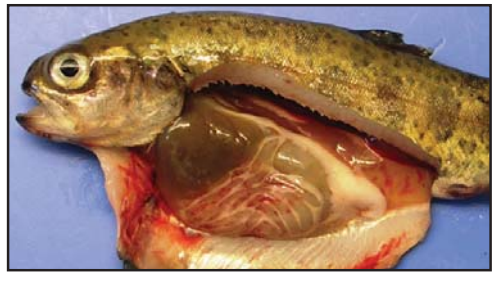

Figure 87. Extensive haemorrhages on the swimbladder, pyloric caeca, intestine and peritoneum

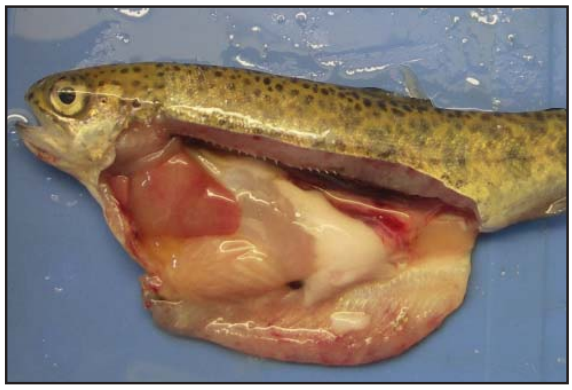

Figure 88. Anaemia, pale liver with haemorrhages, haemorrhages on the intestine and in the dorsal muscle

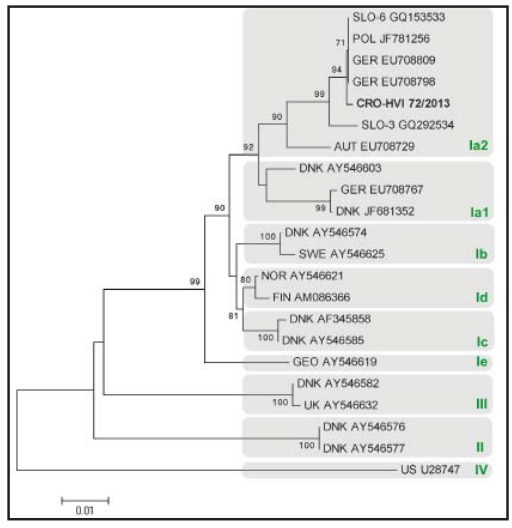

Figure 89. Phylogenetic analysis places Croatian isolate into genotype Ia, subtype 2, subclade Pol II

5.3 Infectious haemopoietic necrosis (IHN)

Infectious haemopoietic necrosis (IHN) is viral disease of rainbow trout and other salmonids caused by an RNA virus of the genus *Novirhabdovirus* in the Family Rhabdoviridae. The virus is related to the VHS virus, is serologically very homogeneous, and can be identified using mono- and polyclonal antibodies. IHNV isolates are grouped based on the partial glycoprotein (G) gene sequences, and there are three major IHNV genogroups in North America designated as U, M and L for the upper, middle and lower portions of IHNV geographical range in North America. The Japanese and Korean isolates constitute a new Japanese rainbow trout genogroup. All European isolates belong to the M genogroup (Figure 95).

The disease has been spread through the United States of America, Canada and Japan and in 1987, was recorded in the French Republic and the Italian Republic and from that time, it was spread to other European countries. Northern pike and some salmonid species are more resistant to the disease, but they can be inapparent carriers. Reservoirs of IHNV are clinically infected fish and covert carriers among cultured, feral or wild fish. Viral particles are shed via urine, sexual fluids and from external mucus, whereas the kidney, spleen and other internal organs are the sites where the virus is most abundant during the course of overt infection. Usually disease appears at a water temperature of between 10°C and 12°C.

The incubation period under farm conditions is from one to three weeks depending on fish age, virus concentration and water temperature, but it can last up to three months.

The clinical signs in trout and salmon fry include haemorrhages on the yolk sac, around the eyes, on the abdomen and on the fin bases. Mortalities in juveniles (Figure 94) can rise up to 80 percent, while in larger fish they rarely exceed 20 to 30 percent. Diseased fish are usually dark pigmented with pronounced exophthalmia and distended abdominal wall (Figure 91); they are lethargic, anorexic and swim close to the water surface (Figure 95). Whitish pseudocast (consisting of faeces and epithelial cells from the intestine) hanging from the anus is often visible. Necropsy reveals pale organs with petechial haemorrhages in the visceral fat and musculature. Intestines are filled with gelatinous content. Older ages display milder clinical signs such as haemorrhages on the fin bases and heart, liver and intestine (Figures 91, 92 and 93). Surviving fish often have spinal deformities.

As for VHS, preliminary diagnosis is based on the epidemiological data, clinical appearance of the disease and absence of bacterial septicaemic infections. Attention should be payed to differentiate IHN from other bacterial or viral septicaemic diseases.

The "gold standard" for the diagnosis of IHN is isolation of the virus on cell culture (EPC, FHM, BF-2 or RTG-2) and subsequent identification using ELISA test, IFAT or VN. Molecular methods like RT-PCR or real-time PCR (qRT-PCR) and gene sequencing are very suitable for virus identification and typing (Figure 95).

Figure 90. Dark pigmentation; haemorrhages on the fat tissue, in the musculature and peritoneum

Figure 91. Haemorrhages on the swimbladder, intestine, pyloric caeca and fat tissue

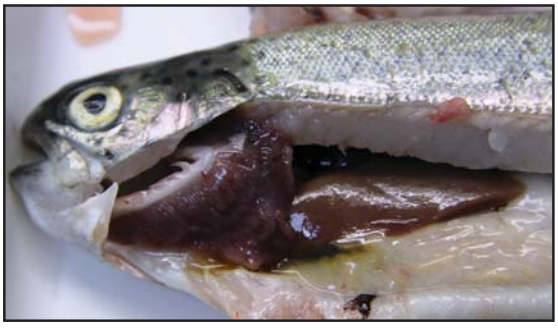

Figure 92. Anaemia, haemorrhages in the gills

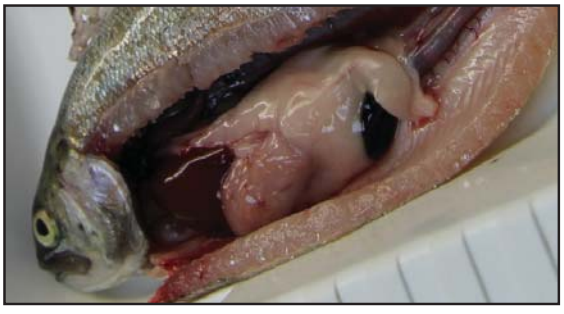

Figure 93. Splenomegaly

Figure 94. Dark pigmentation and exophthalmia are usually the first clinical signs in the infected facility (Photo courtesy of Prof Dr Vlasta Jenčič)

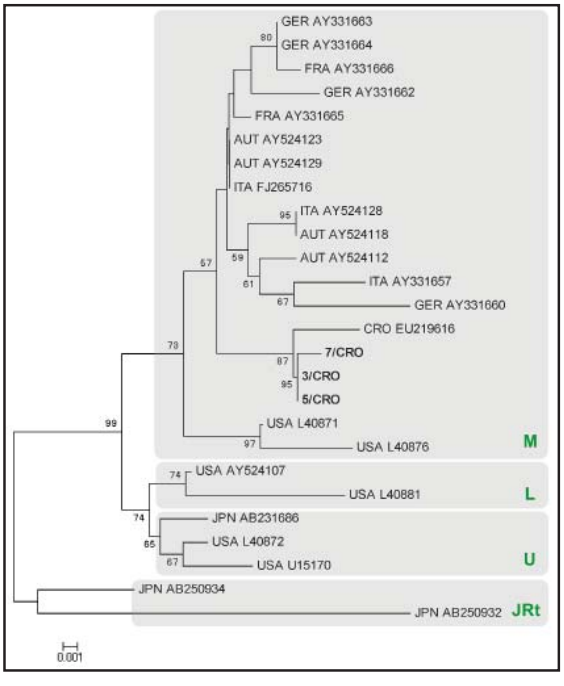

Figure 95. Phylogenetic analysis of Croatian isolate of Infectious haemopoietic necrosis virus

5.4 Koi herpesvirus disease (KHVD)

Koi herpesvirus disease (KHVD) is a herpesvirus infection inducing a contagious and acute viraemia in common carp and varieties such as koi and ghost carp. The causative agent is koi herpesvirus (KHV), which belongs to the Family Alloherpesviridae, cyprinid herpesvirus 3 (CyHV-3). Other herpesviruses known as fish pathogens are CyHV-1, the causative agent of carp pox and CyHV-2, the causative agent of goldfish haematopoietic necrosis.

The disease was first reported from the State of Israel and the Federal Republic of Germany in the late 1990s, and has since been spread globally, predominantly with the trade in koi carp. KHVD affects common carp (*Cyprinus carpio*) and its varieties such a koi, ghost carp and their hybrids. KHV is present throughout Europe, including the United Kingdom of Great Britain and Northern Ireland, and also occurs in Asia and the United States of America. During a KHVD outbreak, there is a noticeable increase of mortality in affected populations. All age groups of fish appear to be susceptible, although under experimental infection, fish up to one-year old are more susceptible.

The most consistent gross pathology is seen in the gills, and this can vary from pale necrotic patches to extensive discolouration, severe necrosis and inflammation (Figures 97 and 98). Another commonly observed gross pathological change is pale, irregular patches on the skin associated with excess mucus secretion (Figure 96) and also with under-production of mucus, where patches of skin have a sandpaper-like texture. Commonly reported clinical signs include anorexia, enophthalmia (sunken eyes), superficial haemorrhaging on the skin and fin bases and fin erosion. Morbidity of affected populations is up to 100 percent, while mortality is usualy 70 to 80 percent and in severe outbreaks can rise up to 90 or 100 percent.

Suspicion that fish are affected with KHV is well founded if typical clinical signs of KHVD are present in susceptible fish species OR typical pathohistological changes are noticed in tissue sections (e.g. necrotic changes and intranuclear inclusions in infected cells) (Figure 99) OR if antibodies to KHV have been detected.

For confirmation of KHV, the “gold standard” is PCR. For surveillance, real-time PCR should be used as the most sensitive and specific test that minimizes the risk of cross-contamination. The most commonly used assay is the Gilad Taqman real-time PCR assay. For final confirmation, PCR products of the correct size should be identified as KHV in origin by sequence analysis.

The ELISA methods have low sensitivity, and may be suitable for detection of the high levels of KHV found in clinically diseased fish tissue, but they are not suitable for KHV surveillance in healthy populations.

Figure 96. Pale, irregular patches on the skin associated with excess mucus secretion, superficial haemorrhaging (Photo courtesy of Dr Agus Sunarto)

Figure 97. Sunken eyes with haemorrhages, pale necrotic patches to extensive discolouration, severe necrosis and inflammation of the gills

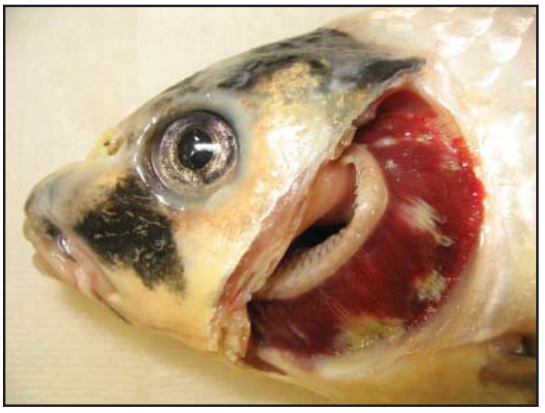

Figure 98. Severe gill necrosis and inflamation of the gills of koi carp (Photo courtesy of Dr Olga Haenen)

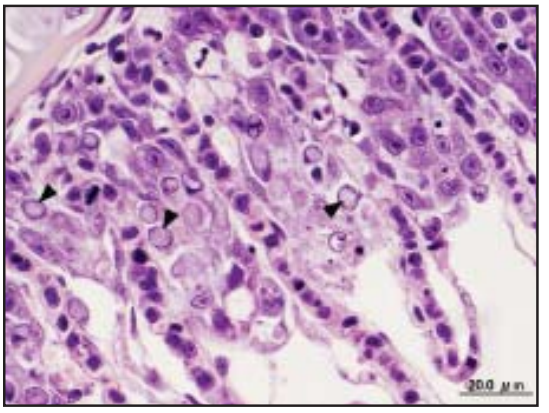

Figure 99. Histo-section of carp gill infected with Koi herpes virus. Note vacuolization and intracellular inclusions in the infected cells (arrows) (Photo courtesy of Dr Miwa Satoshi)

5.5 Spring viraemia Of carp (SVC)

Spring viraemia of carp (SVC) is an acute haemorrhagic and contagious viral infection of cyprinids, mainly common carp, in which the disease usually breaks in spring and causes mortality in juveniles and adults. The disease was described by Fijan *et al.* (1971) when they realized that the virus isolated from affected carp was the causative agent of the syndrome that had previously been known as acute infectious dropsy of carp. The aetiological agent of SVC is *Rhabdovirus carpio* (RVC) or spring viraemia of carp virus (SVCV), which belongs to the genus *Vesiculovirus,* Family Rhabdoviridae. Overt infections were recognized in common carp, grass carp, bighead carp, silver carp, Crucian carp (*Carassius carassius*), goldfish (*Carassius auratus*), tench (*Tinca tinca*) and wels catfish. The disease was described in those European and Asian countries that experience low-water temperatures during winter; however, there have also been recent outbreaks in the United States of America and the United Kingdom of Great Britain and Northern Ireland.

Generally, fish aged up to one year are most susceptible to clinical disease, but all age groups can be affected. Fish become sluggish, gather at the water inlet or sides of the pond, and may lose equilibrium, and finally a noticeable mortality increases in the population. Diseased fish are usually darker pigmented. Typical clinical signs include exophthalmia; pale gills; haemorrhages on the skin, base of the fins and vent; abdominal distension or dropsy and a protruding vent, often with trailing yellowish mucoid fecal casts. All these clinical signs may not be present in individual fish nor in the affected population. There may be a sudden onset of mortality without clinical signs. Gross pathological changes include excess ascitic fluid in the abdominal cavity, usually containing blood, degeneration of the gill lamellae and inflammation of the intestine, which contains mucus instead of feed. Oedema and haemorrhages of the visceral organs are commonly observed. Focal haemorrhages may be seen in the muscle and fat tissue, as well as in the swimbladder (Figure 100).

Preliminary diagnosis is based on the epidemiological data, clinical appearance of the disease and absence of bacterial septicaemic infections. Attention should be payed to differentiate SVC from other bacterial or viral septicaemic diseases.

The “gold standard” for diagnosis of SVC is isolation of the virus on the EPC, FHM or BF-2 cell lines (Figure 101) and subsequent identification using ELISA test, IFAT or VN. Molecular methods like RT-PCR or RT-qPCR followed by sequence analysis are very suitable for virus identification.

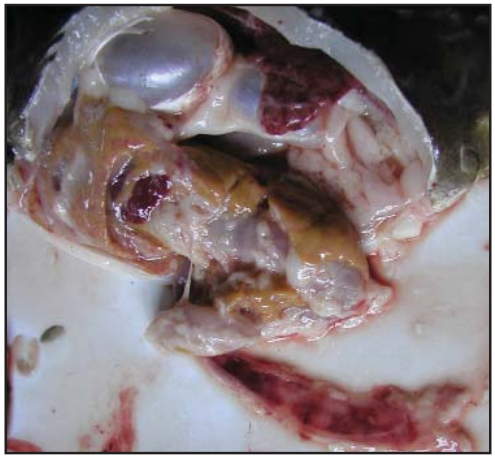

Figure 100. Haemorrhages in the kidney and swimbladder and pale liver in carp infected by Spring viraemia of carp virus

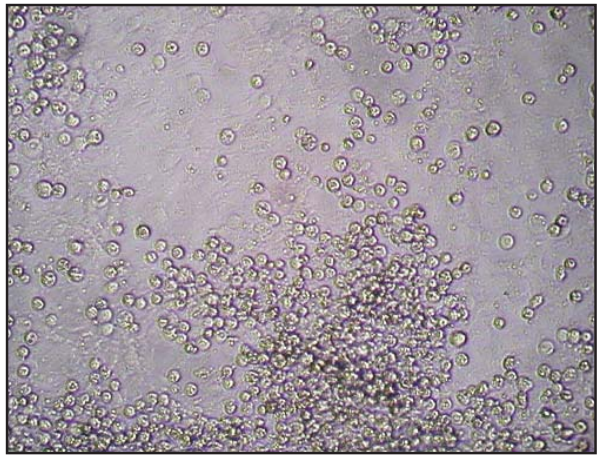

Figure 101. Cytopathic effect in EPC cell lines caused by *Rhabdovirus carpio* (20x)

5.6 Infectious pancreatic necrosis (IPN)

Infectious pancreatic necrosis (IPN) is a highly contagious viral disease affecting fry of salmonid species held under intensive rearing conditions. The disease most characteristically occurs in rainbow trout, brook trout, brown trout, Atlantic salmon (*Salmo salar*), and several Pacific salmon species (*Oncorhynchus* spp.). Susceptibility generally decreases with age, with resistance to the disease being reached at 1 500 degree-days (value obtained by multiplying the age in days by the average temperature in degrees centigrade) except for Atlantic salmon smolts.

The causative agent, infectious pancreatic necrosis virus (IPNV), is a bi-segmented double-stranded RNA virus belonging to the Family Birnaviridae. The disease is transmitted both horizontally via the water route and vertically via the egg. Surface disinfection of eggs is not entirely effective in preventing vertical transmission.

Initially described in brook trout, IPN has now been confirmed in most salmonids throughout the world, as well as in many non-salmonids and shellfish. Several serotypes have been confirmed.

The first sign in salmonid fry is a sudden and usually progressive increase in daily mortality, particularly in the faster-growing individuals. Clinical signs include dark pigmentation, a pronounced distended abdomen (Figure 102) and a corkscrewing/spiral-swimming motion. Cumulative mortalities may vary from less than 10 percent to more than 90 percent, depending on a combination of factors such as virus strain, host and environment. Internally, the fish can display swollen intestine and catarrhal exudates in the lumen. There may also be petechiae on the caecal fat and a pale liver.

Focal necrosis of the acinar pancreatic tissue with necrotic areas replaced by a loose fibrous network and fat degeneration are present (Figure 103). Macrophages and leucocytes may infiltrate pancreatic and hepatic tissues. There may be necrosis and sloughing of the caecal endothelium.

The “gold standard” for the diagnosis of IPN is isolation of the virus on the cell culture (EPC, FHM, BF-2 or RTG-2) and subsequent identification using ELISA test, IFAT or VN. Molecular methods like RT-PCR or RT-qPCR and gene sequencing are very suitable for virus identification and typing.

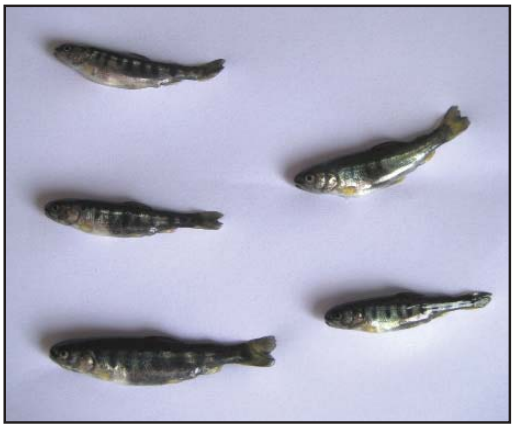

Figure 102. Rainbow trout fry infected with Infectious pancreatic necrosis virus (note dark pigmentation, distended abdomen)

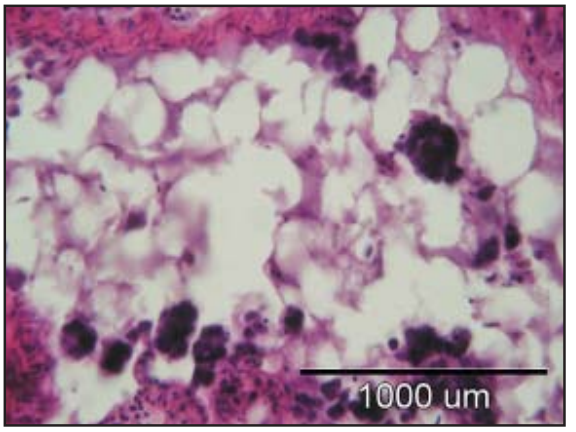

Figure 103. Necrotic changes in pancreas of Infectious pancreatic necrosis virus-positive rainbow trout fry *(Photo courtesy of Prof Dr Ivona Mladineo)*

5.7 Viral encephalopathy and retinopathy (VER)

Viral encephalopathy and retinopathogy (VER), also known as viral nervous necrosis (VNN) is a serious viral disease characterized by significant losses due to vacuolation lesions in the central nervous system and retina of the eye. More than 40 marine and several freshwater fish species from Australia, Asia, North Africa, the United States of America, Canada and Europe have been reported as infected. The causative agent is a small, non-enveloped, icosahedral, single-stranded RNA virus belonging to the genus *Betanodavirus* from the Family Nodaviridae. Currently there are five genotypes based on phylogenetic analysis: a) striped jack nervous necrosis virus (SJNNV), described in species from the Mediterranean, Tahiti and Taiwan POC; b) barfin flounder nervous necrosis virus (BFNNV), reported from Canada, Japan and the Kingdom of Norway; c) tiger puffer nervous necrosis virus (TPNNV), from Japan; d) redspotted grouper nervous necrosis virus (RGNNV), reported from the Kingdom of Spain and Japan; and e) turbot nervous necrosis virus (TNNV), reported from the Kingdom of Norway. Each of those genotypes affects different fish species and requires a different temperature range for propagation in susceptible species. In Mediterranean species, SJNNV and RGNNV, as well as their reassortant genotypes were detected. Susceptible species are European seabass, turbot, sharpsnout seabream (*Diplodus puntazzo*), meagre (*Argyrosomus regius*), red drum (*Sciaenops ocellatus*), Atlantic halibut (*Hippoglossus hippoglossus*), bastard halibut (*Paralichthys olivaceus*), grouper (*Epinephelus* spp.) etc. Reservoirs of infection are infected cultured or feral fish.

The virus can survive outside of the host, in the marine environment for more than year, and in frozen fish for a very long period; however outside of water, it survives less than ten days. It enters the fish through the epithelium or digestive system and due to neurotropism, replicates in the spinal cord, medula oblongata, brain and retinal tissue, causing vacuolization of nerval cells, causing swimming disorders. All age categories are affected, but the highest mortalities are recorded in the larval stage (80 to 100 percent), while mortalities in fry range from 10 to 50 percent, and in older fish can reach 10 percent of the affected population.

Diseased fish swim spirally or assume a vertical position in the water column (Figure 104), show flexing of the body, have swimbladder hyperinflation and in later phases, eye opacity (Figure 106), blindness, mouth reddening (Figure 107) and traumatic lesions could be observed (Figures 105). By necropsy, swimbladder hyperinflation and congestion and haemorrhages of the brain are visible (Figure 108).

Swimming abnormalities are the first sign of an outbreak and together with favourable environmental conditions (high temperature) can justify suspicion of infection with VER/VNN. Isolation of virus on SNN-2 cell lines and RT-PCR are appropriate methods for confirmation of infection. Histologically, vacuolization of the brain and retina of the eye are typical findings (Figure 109).

Figure 104. Abnormal swimming behaviour of European seabass fry (spiral swimming)

Figure 105. Affected specimens are thin, with lesions on the head and mouth

Figure 106. Corneal opacity is often visible in affected specimens

Figure 107. Lesions and haemorrhages on the mouth and head

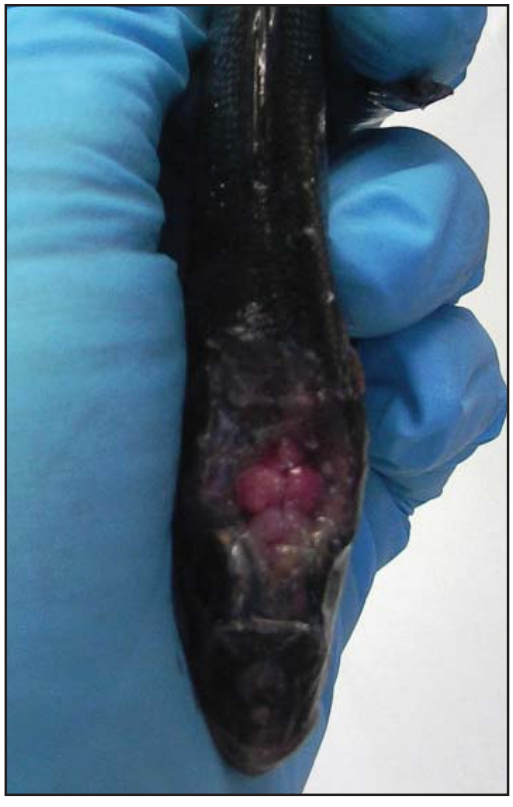

Figure 108. Congestion and haemorrhages on the brain are visible on necropsy

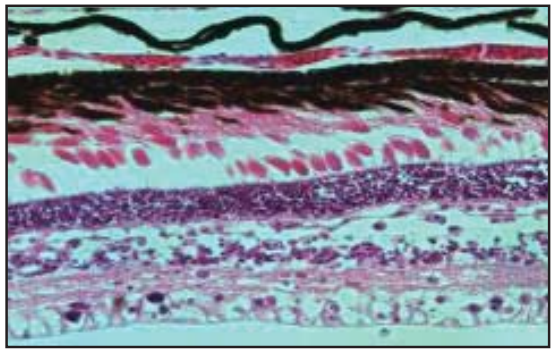

Figure 109. Vacuolization in the retina of fish infected by Betanoda virus (H&E, 40x)

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6. Bacterial diseases

Description of several bacterial diseases have been included in this manual due to their ability to cause haemorrhagic septicaemia and thus the possibility of confusing them with viral septicaemia. Data and knowledge about bacterial diseases should facilitate evaluation of health status at the aquaculture facility. Several other diseases are causing economic losses at the aquaculture facilities in the region and as with other diseases affecting aquaculture facilities, they begin suddenly and progress rapidly, often with high mortality. Knowledge of the most common bacterial diseases affecting the aquaculture industry in the Western Balkan Region will help veterinarians to broaden their expertise, and so actively contribute to aquaculture development.

6.1 Bacterial kidney disease (BKD)

Bacterial kidney disease (BKD) is a serious, slowly progressive, chronic, systemic granulomatous disease of farmed and wild salmonids in both fresh and seawater. It was first described in the early 1930s in wild salmon in Scotland. Salmonids are clinically susceptible, especially members of the genus *Oncorhynchus,* which includes Pacific salmon and rainbow trout. The disease has been reported in North America, Japan, Europe and the Republic of Chile. *Renibacterium salmoninarum*, a Gram-positive bacterium, is the causative agent of BKD.

Affected fish may show no obvious external signs or may show one or more of the following: pale gills, exophthalmia, abdominal distension (due to ascites), skin blisters (filled with clear or turbid fluid), shallow ulcers (the results of broken skin blisters), haemorrhages (particularly around the vent) and more rarely, cavitations in the musculature that are filled with blood-tinged caseous or necrotic material. At necropsy, fluid may be present in the abdomen, varying haemorrhages on the abdominal wall and viscera, a membranous layer on one or more of the visceral organs (Figure 110), and most characteristically, creamy-white granulomatous lesions in the kidney and less frequently in the liver, spleen and heart (Figure 111). Lesions are characterized by chronic granulomatosis, principally of kidney tissue, but extending to the liver, cardiac and skeletal muscle. The granuloma is often large, with a central caseous zone bounded by epithelioid cells and infiltrating lymphoid cells. The presence of a capsule is variable, and lack of encapsulation is often associated with more pronounced infections.

Renibacterium salmoninarum is a small, Gram-positive diplococcus growing at 15°C –18°C and not at all at 25°C. Isolation (2–6 weeks at 15°C) in selective media (i.e. selective kidney disease medium (SKDM), kidney disease medium (KDM-2)) has proved useful in isolating *Renibacterium* from mixed cultures. A monoclonal antibody-based ELISA appears to be effective for the diagnosis of BKD. IFAT and fluorescent antibody technique (FAT) have been developed for the diagnosis of BKD. A further refinement involves concentrating samples on membrane filters, which are used with FAT.

A PCR-invoking region of the gene encoding the 57 kDa soluble protein (p57) is recommended for the screening of broodstock for the presence of BKD. A specific, sensitive real-time PCR has been developed to recognize BKD in kidney tissue. Loop mediated isothermal amplification (LAMP) methods have been proposed as a sensitive and specific method for the detection of *R. salmoninarum.*

There is evidence that under some conditions the pathogen elicits an immune response in fish, and there are some reports of vaccination. The protective ability of the vaccine is questionable, however, and one of the problems is the intracellular nature and vertical transmission of the agent.

Chemotherapy (i.e. erythromycin) provides limited and only temporary relief. Some success at chemotherapy has been reported with erythromycin, sulphonamides, chloramphenicol, penicillin, clindamycin, kitasamycin and spiramycin and enrofloxacin. The bacterium can survive and multiply within phagocytic cells. Screening of farmed broodstock and regular testing of growing stock for the agent combined with disinfection and movement controls have proven effective in Europe.

Figure 110. Infected rainbow trout exhibiting haemorrhages on the liver, granulomatous lesions on the kidney, and splenomegalia

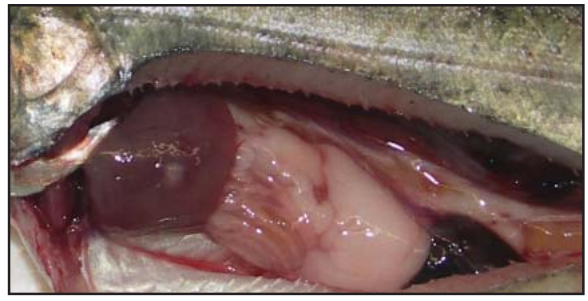

Figure 111. Granulomatous lesions in the liver and kidney of rainbow trout (Photo courtesy of Dr Dražen Oraić)

6.2 Yersiniosis (enteric redmouth disease – ERM)

Yersiniosis is bacterial septicaemic disease of farmed salmonids. The term "yersiniosis" is synonymous with enteric redmouth disease (ERM), a disease of fish caused by overt infection with Yersinia ruckeri, a member of the Family Enterobacteriaceae. ERM was first described in association with losses in rainbow trout in Hagerman Valley, Idaho in the 1950s. It is now enzootic across all the major salmonid farming areas of the world and is widespread in European trout farms where it is primarily a problem of young rainbow trout. All salmonids are susceptible, and outbreaks are confirmed in North America, Europe, the Republic of South Africa, the Republic of Chile and New Zealand. Feral fish, bait fish and even ornamentals have been detected as sources of infection. The causative agent of the disease, Y. ruckeri, is a Gram-negative, non-spore forming, non-encapsulated rod-shaped bacterium, with variable motility. Yersiniosis most commonly affects rainbow trout of approximately 7.5 cm in length. The disease is less severe but more chronic in larger fish, i.e. those of 12.5 cm in length. Severity peaks with a water temperature of 15°C–18°C and decreases when it drops to 10°C or below. Overly fat or stressed fish are thought to be more susceptible to severe epizootics. However, the handling of fish and crowded conditions causing excess ammonia and other metabolic waste products in the water with consequential decrease in oxygen levels may precipitate outbreaks of clinical disease. Nevertheless, outbreaks generally occur only after the fish have been exposed to large numbers of the pathogen. If left untreated, cumulative losses may account for as much as 30 to 80 percent of the rainbow trout population.

Gross external signs are lethargy, skin darkening and congestion around the mouth and opercula, in the mouth and at the base of the fins (Figure 113). Other clinical signs include exophthalmia, ulceration and cutaneous petechiae. Internally, the fish show signs of haemorrhagic septicaemia, with congestion and petechiae throughout the peritoneum and visceral organs, in particular the caecal fat. Splenomegaly and a fluid-filled stomach and intestine are also observed (Figure 112). Necrosis of the haematopoietic tissues in the kidney and spleen following bacterial invasion are most commonly seen, and bacteria may be observed in any organ throughout the body. With bacterial spread to the gills, musculature and liver, there is capillary dilation and haemorrhage, tissue oedema and further necrosis.

Gross and histological signs are helpful in diagnosis, but confirmation requires isolation on general nutrient agar (24 hours at 22°C) such as TSA or on the selective media (Figure 114 and Figure 115), and identification. IFAT has a proven track record with the diagnosis of yersiniosis. A PCR was successful in detecting *Y. ruckeri* in diseased trout tissues, and LAMP, amplifying the specific genes, is regarded as tenfold more sensitive than PCR.

Broad-spectrum antibiotics are effective in controlling an outbreak, but increasing antibiotic resistance is observed and sensitivity should be tested.

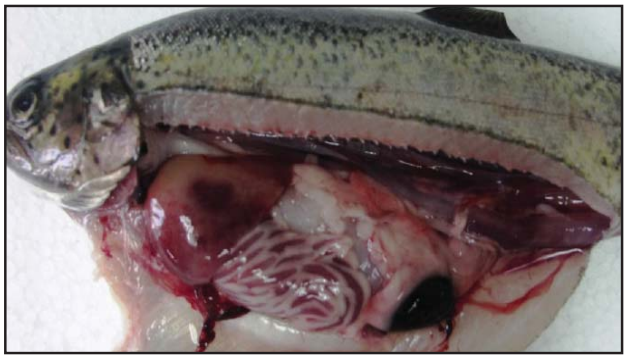

Figure 112. Petechial haemorrhages on the liver and pyloric caeca, and splenomegaly in infected rainbow trout

Figure 114. Pure bacterial colonies on blood agar

Figure 113. Haemorrhages around and in the mouth (Photo courtesy of Dr Dražen Oraić)

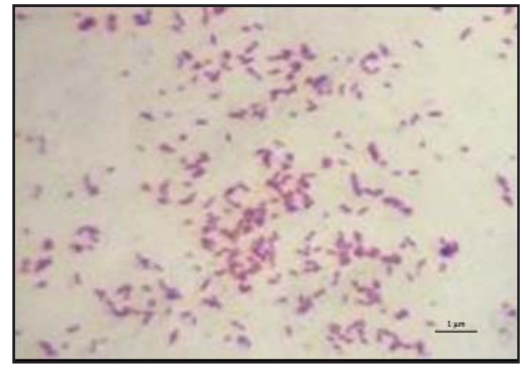

Figure 115. Short, Gram-negative rods, *Yersinia ruckeri* (100x)

6.3 *Aeromonas* infections

6.3.1 Motile Aeromonas septicaemia (MAS)

Motile *Aeromonas* septicaemia (MAS) is a common bacterial disease of cyprinids; however, all freshwater fish are susceptible. MAS has been associated with several members of the genus *Aeromonas*, which are ubiquitous in freshwater environments. The most important fish pathogen is *A. hydrophila*, and members of this group are often referred to as the *A. hydrophila* complex. *Aeromonas hydrophila* is a ubiquitous organism that occurs naturally in most freshwaters of the world. It is capable of living and proliferating in any water containing organic enrichment. The bacterium usually causes disease only in fish that are immuno-compromised by environmental stressors, or when they have suffered some mechanical or biophysical injury. *Aeromonas* is also commonly isolated from the mucosal surfaces and internal organs of clinically healthy fish. The highest prevalence of disease outbreaks is in organically polluted waters. Ingestion of contaminated feed may also be a source of infection. Predisposing risk factors include stressful environmental conditions such as high temperature, intensive breeding, overcrowding, organic pollution, high levels of ammonia and nitrite, sudden changes in pH and hypoxia. Motile aeromonads often invade skin wounds caused by water molds or ectoparasites. Spawning, rough handling and transport may also provoke the occurrence of disease.

Although rare, due to the diversity of strains of *A. hydrophila* with regard to virulence or pathogenicity, this bacterium may cause high mortality among cultured fish without the presence of stressful influences.

Clinical signs of disease range from superficial to deep skin lesions, to a typical, Gram-negative bacterial septicaemia, with or without skin lesions. Skin lesions include variously sized areas of haemorrhage and necrosis on the skin and at the base of the fins which may progress to reddish or gray ulcers with necrosis extending to the musculature. Ulcers may progress to haemorrhagic septicaemia, with exophthalmia, lepidorthosis, a distended abdomen that has serosanguinous fluid, visceral petechiation, and a haemorrhagic and swollen lower intestine and vent. Anorexia and dark colour are most common with systemic disease (Figure 116).

Definitive diagnosis of motile aeromonad infection requires biochemical identification of clinically significant numbers of the suspect bacterium in target tissues, with attendant clinical signs. Isolates (Figure 117) vary widely in antigenicity, making immunological identification difficult.

6.3.2 Aeromonas salmonicida infections

The term "furunculosis" is used to describe a lethal septicaemia of salmonid species caused by the bacterium *Aeromonas salmonicida* subsp. *salmonicida*, but can also denote infections with atypical strains of the furunculosis bacterium occurring in a broad range of freshwater and marine species and most frequently manifesting with severe skin ulceration and wounds.

In the context of fish disease, the term furunculosis may be used in a broad sense, denoting a variety of disease manifestations caused by bacteria belonging to the species *Aeromonas salmonicida*, occurring in a wide range of freshwater and marine fish species. Many of these infections, however, fail to display the lead clinical sign that originally gave the disease its name (furuncles: haemorragic necrosis of skeletal muscle), and in the following, distinction will be made between classical furunculosis, a disease occurring predominantly in salmonids that is caused by *A. salmonicida* subsp. *salmonicida*, and disease conditions arising from infection with atypical *A. salmonicida* strains (*A. salmonicida* subspp. *achromogenes, masouicida, smithia* or *pectinolytica*), which are described from both salmonids and non-salmonids, and from numerous other freshwater and marine species.

The clinical pattern of classical furunculosis is that of a septicaemic infection with high mortality, chronic disease signs in survivors, little to no development of natural immunity, and recurrency of outbreaks most frequently from spring through late autumn and early winter. Clinically, the acute form or the disease is manifested by generalized septicaemia accompanied by melanosis, inappetence, lethargy and haemorrhages on the fin bases. The subacute form is characterized by lethargy, slight exophthalmia, haemorrhages in the muscle and other tissues and bloody discharge from the nares and anus. Necropsy reveals haemorrhages on the liver, swelling of the spleen, and kidney necrosis. The mortality rate is usualy low; diseased fish survive but the survivors have scars.

In addition to furunculosis, there are some other disease conditions caused by *A. salmonicida*. One of them is carp erythrodermatitis (CE) caused by an atypical strain of *A. salmonicida*, a Gram-negative, rod-shaped organism isolated from the skin lesions of common carp. CE is a subacute to chronic contagious disease with variable morbidity and mortality. Infection often starts after mechanical injury of the skin, and a haemorrhagic inflammatory process develops between the epidermis and dermis leading to the formation of an ulcer (Figure 118). Darkened colouration of the skin and inappetence are the most typical clinical signs of the disease. Sometimes secondary bacterial or fungal infections are complicating the disease. Sometimes infection may result in generalized septicaemia and death. If affected fish recover, it is possible to notice healed ulcer-like greyish scars (Figure 119). Unlike furunculosis, which occurs at water temperature under 16 ^{o}C, CE occurs independently of water temperature.

Typical clinical signs should be confirmed by isolation of the causative agent on a general nutrient agar such as TSA or on selective media, and identification. Occurrence of colonies surrounded by a dark-brown water-soluble pigment after incubation at 20–25^{o}C for 3 to 4 days is indicative for *A. salmonicida*. Isolation should be followed by identification.

Figure 116. Carp infected with Motile *Aeromonas* septicaemia

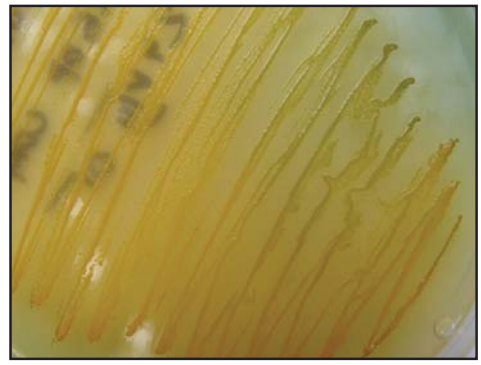

Figure 117. Colonies of *Aeromonas hydrophila* isolated from diseased carp

Figure 118. Lesions on the body trunk of common carp typical for Carp erythrodermatitis

Figure 119. Healed ulcer forming a scar

6.4 Vibriosis

Various *Vibrio* infections caused by the Gram-negative bacteria *Vibrio anguillarum, V. salmonicida*, *V. ordalii* and *V. vulnificus* are among the diseases that lead to the biggest losses in aquaculture all over the world. *Vibrio* species are ubiquitous in aquatic ecosystems, they can survive in seawater,and they are thermo-dependent. Vibrioses are serious diseases of fish, crustaceans and shellfish, while some of the *Vibrio* spp. are of potential importance to humans, causing risk of zoonoses in aquaculture professionals and consumers of aquaculture products. Vibriosis caused by *V. anguillarum* affects many marine fish species, including European seabass, meagre, European seabream and flathead grey mullet (*Mugil cephalus*), and also in rainbow trout cultured in sea and brackishwater. A few decades ago, there were outbreaks of vibriosis of rainbow trout cultured in freshwater after feeding with fresh marine fish. Vibriosis caused by *V. salmonicida* causes “hitra disease” or coldwater vibriosis. Both diseases are characterized by haemorrhagic septicaemia. Vibriosis caused by *V. anguillarum* serotype O1 is a limiting factor in European seabass culture in the Mediterranean region and is still among the most damaging diseases affecting Croatian aquafarms (Figure 120), with total mortalities ranging from 20 to 50 percent during the ongrowing period, if proper treatment is not applied.

Epizootiological studies have revealed that environmental conditions preceding outbreaks of vibriosis are usually a rapid increase or decrease of seawater temperatures from 17°C to 19°C and inversely. However, there are also acute outbreaks occurring in summer, while in winter, chronic forms of vibrosis appear. All age categories are affected by vibriosis, and juveniles are the most susceptible. The disease is characterized by extensive haemorrhages on the fin bases, fins, skin and liver (Figure 120); in the subacute form, haemorrages occur on the stomach and intestine (Figure 121 and 123), accompanied by ulceration in the chronic form of the disease (Figure 122).

Vibrio vulnificus can be pathogenic for humans, shrimp and fish, and this bacterium is very heterogeneous, comprising three biotypes and more than nine serovars with a worldwide distribution. Biotype 2 is the only biotype related to epizootics of fish, mainly eel, where it causes haemorrhagic septicaemia with external ulcers and jaw erosion.

Isolation of *Vibrio* spp. from fish can be done on marine agar, brain heart infusion agar (BHIA), TSA supplemented with 5 percent (v/v) blood and NaCl at 0.5 to 3.5 percent (w/v), and on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Figure 124) with incubation at 15°C –25°C followed by species identification by biochemistry. Biotyping and serological tests (immunoblotting or agglutination tests) are used to distinguish between pathogenic and non-pathogenic bacteria. PCR methods for the identification of the most common species have been developed. Outbreaks of vibriosis are usually controlled by antibiotic (potentiated sulphonamides, oxytetracycline, flumequine, florfenicol) treatments via feed, which should be performed according to the results of an antibiogram. Vaccination of European seabass against *V. anguillarum* serotype O1 is effective, by immersion in hatchery followed by revaccination (Figure 125), either by immersion or injection. In practice, to avoid the use of antibiotics all seabass fingerlings should be injected with *Vibrio* vaccines prior to ongrowing.

Figure. 120. Haemorrhages on the head, opercula and the fin bases in the acute form of vibriosis

Figure 121. Subacute form of vibriosis with more pronounced haemorrhages on the mouth, head, fin bases, fins and abdominal wall

Figure 122. Chronic form of vibriosis characterized by skin ulceration

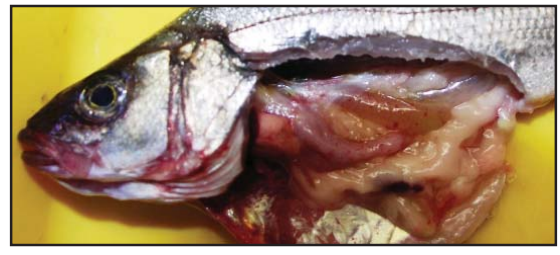

Figure 123. Necropsy reveals haemorrhages on the liver, stomach and intestine (subacute to chronic course)

Figure 124. Growth of *Vibrio anguillarum* (yellow colonies) on selective, TCBS agar

Figure 125. Revaccination of European seabass fry against *Vibrio anguillarum* by immersion

6.5 Infections with "yellow-pigmented bacteria"

This group of diseases is caused by bacteria from the genus *Flavobacterium* (formerly known as *Cytophaga* or *Flexibacter*) in freshwater fish and *Tenacibaculum* (formerly known as *Flexibacter*) in marine fish. All these bacteria form yellow to yellowish-brown or orange-pigmented colonies when grown on solid media. Their unique characteristic is gliding motility, which differs from ordinary flagellar motility. Bacteria from these genera are Gram-negative rods with flexus filaments. There are three *Flavobacterium* species that are primary pathogens of cultured and wild freshwater fish: *F. columnare*, the cause of columnaris disease, *F. branchiophilum*, the cause of bacterial gill disease, and *F. psychrophilum,* the cause of bacterial coldwater disease. The diseases and the associated mortalities caused by them are of the broadest host and geographic ranges.

6.5.1 Bacterial coldwater (BCW) disease

Bacterial coldwater (BCW) disease is caused by *F. psychrophilum* and affects a broad range of salmonid and non-salmonid fish species. It occurs at water temperatures below 16°C and is most prevalent and serious at 10°C. All ages of fish are affected, but fry and fingerlings are particularly vulnerable to infections. The main clinical signs are characteristic open lesions leading to necrosis on the external body surfaces of fish (Figure 128). The pathology and clinical signs can include loss of appetite, eroded fin tips and the appearance of white areas on the fins. In some instances, exophthalmia, abdominal distension with increased volumes of ascites and pale gills (Figure 126) are visible. In advanced cases, necrosis of the caudal region may be severe and progress until the caudal vertebrae are exposed. Rainbow trout fry syndrome (RTFS) is another disease manifestation caused by *F. psychrophilum* that affects the early life-stage of fish. In its acute form, mortalities of up to 50 percent or more of any affected stock may occur. The main features of the disease are lethargy, exophthalmia (often bilateral), dark skin pigmentation and pale gills. Necropsy reveals anaemic and pale kidney and liver with ascitic fluid in the body cavity causing distention of the abdominal wall (Figure 127). The disease is transmitted horizontally due to the ability of this bacterium to survive and maintain its infectivity for a long time in fish, benthic organisms, and algae in the water column. There is also evidence of vertical trasmission of the disease, and bacteria have been isolated from ovarian fluid, milt and the surface of eggs.

A successful diagnosis requires all available information, such as disease history, rearing conditions, water temperature and the presence of characteristic clinical signs, followed by detection of typical bacteria in smears and bacterial isolation and biochemical identification. Ordinary bacteriological media are not appropriate for primary isolation of bacteria from the tissue, therefore *Cytophaga* medium was developed to support the growth of bacteria with reduced nutrient requirements. Molecular methods like PCR and sequencing have been developed to detect pathogen in infected tissues.

6.5.2 Columnaris disease

Columnaris disease is caused by another species of the genus *Flavobacterium*, *F. columnare*. The disease is distributed wordwide in freshwater fish, including cultured carp, channel catfish, goldfish, eel, perch, salmonids and tilapia. Losses caused by this bacterium can be very high, especially in the channel catfish industry. The name comes from the fact that upon examination of wet-mount preparations of the typical lesions, column-like structures formed by bacterial colonies are evident (Figure 129). These bacteria are, like other members of the genus, thin Gram-negative rods that are motile on agar media by flexing motion. The reservoirs of infection are freshwater fish, eggs and rearing water. The gills are the main site of release of the pathogen. Bacteria can survive for up to 16 days in water loaded with organic matter at 25°C.

Columnaris is an acute to chronic disease affecting primarily the gills, skin and fins. Usually death occurs before the appearance of gross external lesions. In subacute or chronic course in adult fish, yellowish areas of necrotic tissue appear in the gills, resulting in complete gill destruction. On the skin, small zones of lesion with a distinct reddish tinge appear first at the base of the dorsal fin, followed by fin deterioration, with the lesion starting from the fin base and progressing to the outer edge and extending laterally, causing "saddle back disease". Lesions, usually covered with yellowish mucus, can appear elsewhere on the skin

and progress to the deeper layer. Progressive gill necrosis causes impaired breathing, exhaustion and finally death of fish.

Early detection of the pathogen is very important to prevent spreading of the disease. Isolation of *F. columnare* from external lesions is possible on low-nutrient media (*Cytophaga* agar), while no growth occurs on TSA, NA or MA. Besides isolation, there are a few rapid methods such as LAMP, PCR and Taqman-based real-time PCR available to detect the pathogen in tissue.

Management plays a key role in prevention of columnaris. Optimal feeding, low stocking density and good water quality are elements important to prevent the disease. Different chemical treatments are successful in controlling the disease; these include bath in copper sulphate, potassium permanganate or hydrogen peroxide or the administration of antimicrobials (e.g. oxytetracycline or potentiated sulphonamides) in feed. However, if antimicrobial treatment is not started at the proper time, the results are questionable.

Figure 126. Chronic course of bacterial coldwater (BCW) disease in brook trout with gill destruction (Photo courtesy of Dr Dražen Oraić)

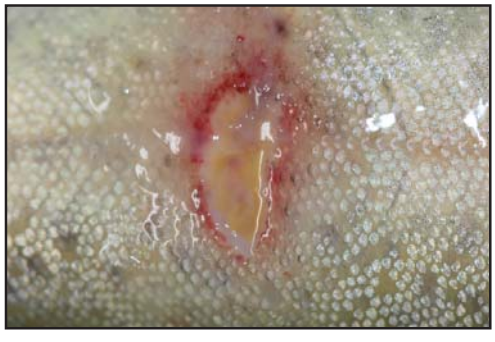

Figure 128. Lesion on the external surface of rainbow trout due to infection with *Flavobacterium psychrophila* (Photo courtesy of Dr Branko Šoštarić)

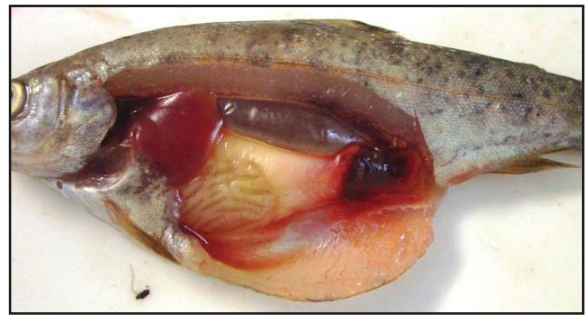

Figure 127. Rainbow trout fry syndrome (RTFS) in rainbow trout fry (note ascites, enlarged spleen)

Figure 129. "Column-like" lesion on the skin of common carp infected with *Flavobacterium columnare* (Photo courtesy of Dr Dražen Oraić)

6.5.3 Tenacibaculum maritimum

Tenacibaculum maritimum is the aetiological agent of an ulcerative disease known as tenacibaculosis which affects a large number of marine fish species, causing significant losses in marine aquaculture. This bacterium belongs to the Family Flavobacteriaceae, and was recently reclassified from the genus *Flexibacter* to *Tenacibaculum.* The disease was first described in cultured marine fish in Japan and nowdays is found all over the world. It causes great economic losses in marine fish culture in the Mediterranean area, the disease affecting turbot, European seabass, gilthead seabream and many other wild fish species. Bacterial infection

causes severe necrotic and ulcerative lesions on the skin (Figure 130), mouth, gills (Figure 132), fins, tail and head (Figure 131). Sometimes the disease becomes systemic and is characterized by emaciation, inappetence, intestines filled with liquid content and fibrinous casts (Figure 133). Sometimes the mixed infection of *Vibrio anguillarum* and *T. maritimum* occurs. There is no host specificity, and wild fish can serve as reservoirs of infection. An increased prevalence and severity occurs at higher temperatures and salinity, but more often outbreaks appear during the winter months; they are dependant on the culture conditions (e.g. rearing density, feeding regime, feed quality, water quality).

Presumptive diagnosis is based upon clincial signs including external lesions and microscopic detection of long, tiny, filamentous bacteria in wet mounts of skin scrapings. Isolation of bacteria from infected specimens is difficult; when material is taken from external lesions and cultured on ordinary media, it is overgrown by saprophytic or other bacteria (e.g. *Vibrio* spp.) and therefore, there is a need for specific media which will favour the growth of *T. maritimum*. *Cytophaga* agar with addition of sea water or *Flexibacter maritimum* medium (FMM) are able to support the isolation of *T. maritimum* (Figure 134); further identification by determination of biochemical properties is then required. PCR methods are useful to detect bacteria from diseased tissue or for the identification process (Figure 135). As antibacterial treatments often fail, the approach to disease prevention and treatment should involve improvement of hygienic conditions, the use of good-quality feed, immunomodulation and the prevention of other diseases. Fish vaccinated against vibriosis suffer less losses due infection by *T. maritimum*.

Figure 130. Infected fish are cachectic, with visible scale loss and skin lesions

Figure 131. Pronounced skin lesions on the head

Figure 132. Swollen, anaemic gills

Figure 133. Generalized anaemia, intestine filled with liquid content and fibrinous cast

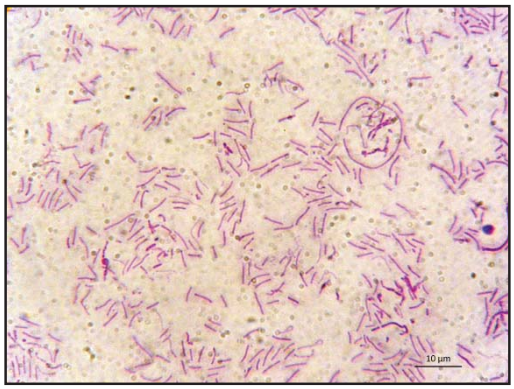

Figure 134. Long Gram-negative rods, *Tenacibaculum maritimum* (100x)

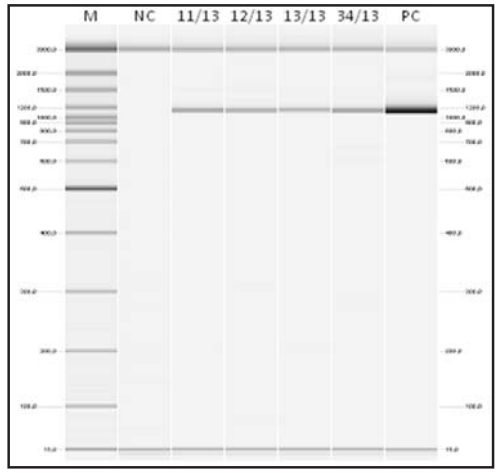

Figure 135. PCR diagnosis of *Tenacibaculum maritimum*

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7. Epizootic ulcerative syndrome (EUS)

Epizootic ulcerative syndrome (EUS) is a disease of fresh and brackishwater fish species, both farmed and wild populations. EUS is caused by the oomycete *Aphanomyces invadans* (=*A. piscicida*). It has a seasonal character and primarily causes problems at temperatures between 18°C–22°C and after periods of heavy rain, conditions that favour sporulation of *A. invadans*. Low temperatures have been shown to delay the inflammatory response of fish to oomycete infection. In affected farms, morbidity is up to 50 percent and mortality can also reach 50 percent of the affected population.

The disease was first reported in Japan and Australia in the early 1970s and has since spread throughout Asia. It was also reported from North America and more recently (since 2007), from Africa (the Republic of Botswana, the Republic of Namibia, the Republic of South Africa, the Republic of Zambia). When the disease is introduced into a population, outbreaks may be seen every year.

Many species of wild and farmed fish are susceptible to the disease, with snakeheads (*Channa*), spiny eel (*Mastacembelus*), barbs (*Puntius*), gourami (*Trichogaster*), catla (*Catla*), mullet (*Mugil*) and labeo (*Labeo*) being the most sensitive. Fish belonging to the genera *Oreochromis* (e.g. tilapia), *Chanos* (e.g. milkfish) and *Cyprinus* (e.g. common and koi carps) are not or only less susceptible.

Figure 136. *Aphanomyces invadans* (= *A. piscicida*) infection in Japanese pond-cultured ayu, *Plecoglossus altivelis* (Photo courtesy of Prof. Kishio Hatai)

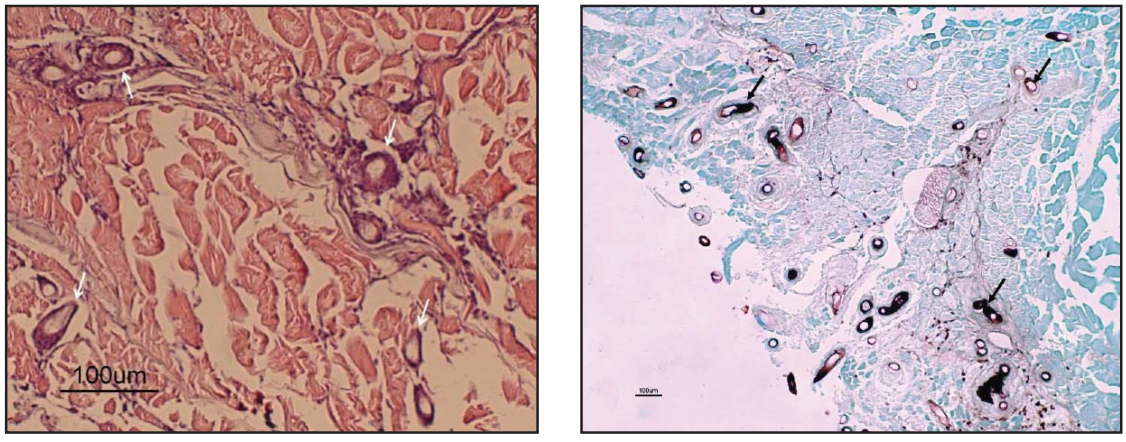

Figure 137. Histopathology of EUS-suspected barb showing: (i) typical mycotic granulomas surrounding the invasive fungal hyphae penetrating into the muscle layer (H&E stain) (left photo); and (ii) typical mycotic granulomas surrounding the invasive fungal hyphae in the skin layer (Grocott's stain) (right photo). Photo courtesy of source FAO (2009).

The appearance of EUS is characterized by sudden high mortality in wild and farmed fish. Usually, a wide range of species and sizes of fish are affected, showing abnormal behaviour and clinical signs of the disease. Typical clinical signs include loss of appetite and floating close the water surface. Red spots or small to large ulcerative lesions are visible on the body surfaces, mainly on the head, opercula and caudal peduncle (Figure 136). In the more pronounced course of the disease, deep erosions and large wounds can be found both in the head and on the body. Histological sections will reveal extensive, serious, mycotic granulomatous changes and distinct flocculent necrotic muscle fibers (Figure 137). The invasive oomycete can grow the whole way through the muscle tissue into the spinal cord, kidney and peritoneum. In late stages, very compact fibromatous stroma containing granulomatous tissue and dead hyphae can be seen. Such fish can survive but are often deformed. By microscopy of a tiny piece of muscle tissue that has been cut from an area deep under a lesion, thick-walled bifurcated hyphae without cross walls can be seen.

Diagnosis is based upon clinical appearance of the disease, and confirmation is performed by isolation of *A. invadans* on glucose peptone (GP)/glucose peptone yeast (GPY) medium or agar with addition of antibiotics. However, sometimes these attempts fail, and thus the most accurate diagnosis is based on detection of DNA of *A. invadans* from actively growing colonies or from the infected tissue.

Control of EUS in natural waters is probably impossible. In outbreaks occurring in small, closed waterbodies, liming of water and improving water quality, together with removal of infected fish is often effective in reducing mortalities and controlling the disease.

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8. Diseases of molluscs

Serious epizootics may endanger mollusc aquaculture, as was experienced in the French Republic in the 1970s and 1980s. Molluscs are cultured in the open sea, and there are no available treatments nor immunoprophylaxis available for their diseases. The best way to prevent the introduction and spread of pathogens of commercially important species is the implementation of good biosecurity and disease surveillance programmes.

The Mediterranean mussel and the European flat oyster are traditionally cultivated species in the Western Balkan Region of the Adriatic coast. Bonamiosis and marteiliosis are OIE and EU-listed diseases, and *O. edulis* is susceptible to both diseases, while *M. galloprovincialis* is susceptible to marteiliosis.

8.1 Marteiliosis

Marteiliosis, also known as "Aber disease", is a disease of molluscs caused by the protistan parasite *Marteilia refringens,* which is a member of the Phylum Cercozoa, Order Paramyxida. The pathogen sporulates in the digestive gland epithelia, causing low condition index, weakening of the animal, emaciation and glycogen depletion. The digestive gland may be become discoloured, growth is disturbed and finally, the animal succumbs, while at the same time, the parasite sporulates, releasing spores to the environment. The spores then enter the intermediate host, a zooplanktonic organism such as the copepod *Paracartia grani,* and the mollusc then becomes infected by ingestion of the intermediate host. Within the mollusc, the parasite develops in the epithelia of the labial palp and digestive gland by internal cleavages when the sea temperature is above 17°C. Mortality usually begins a month after infection. Higher salinity represses sporulation. In general, the mortalities coincide with seasonal changes; the disease begins in May and peaks between June and August, after which there is a decrease over the winter months and the parasite then remains quiescent from February to April.

The most susceptible species is the European flat oyster followed by mussels (*M. edulis* and *M. galloprovincialis*) and cockles (*Cardium edule*). Marteilosis was first detected in the French Republic where it devastated oyster production. It was subsequently reported from the Kingdom of Spain, the Italian Republic, the Portuguese Republic, the Republic of Croatia, the Kingdom of Morocco, the Hellenic Republic and the Republic of Slovenia, and most recently, from the Kingdom of Sweden and the United Kingdom of Great Britain and Northern Ireland. According to characterization of the internal transcribed spacer (ITS) region of the parasite, it is possibe to distinguish three types: O type (most common for oysters); M type (most often in mussels) and C type (found in cockles). Cases of co-infection of oyster or mussels with both M & O types have been reported.

Diagnosis of infected molluscs is made by histology, cytology or the use of molecular techniques. Histological examination of the epithelia of the stomach, intestine and digestive tubules (Figure 139) will show the presence of *M. refringens* in infected animals. Cytological examination can be carried out by the use of imprints (Figure 138). Smears of the digestive gland are fixed, stained and examined microscopically. Molecular techniques such as PCR may be used to confirm the identification of *M. refringens*.

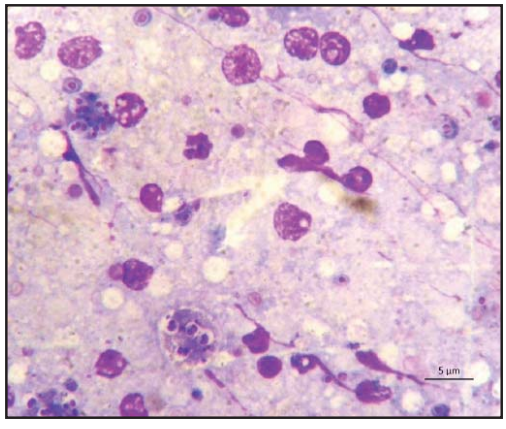

Figure 138. *Martiela refringens* in the imprint of the digestive gland of mussel (*Mytilus galloprovincialis*) stained with Hemacolour kit 40x

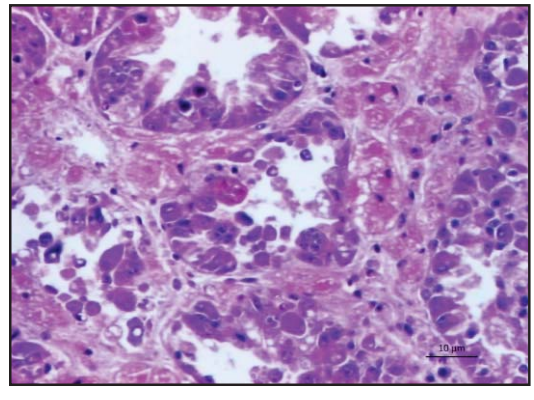

Figure 139. *Martiela refringens* parasiting the digestive gland tubuli of *Mytilus galloprovincialis*. Histological section, H&E, 40x

There is no eradication nor treatment of the disease; thus control should be based on import restrictions and a well-designed surveillance programme.

8.2 Bonamiosis

The protistan parasite *Bonamia ostreae* causes lethal infection of haemocytes in *Ostrea edulis*, affecting both wild and cultivated stocks. Bonamiosis caused a drastic decrease of European flat oyster culture along the Atlantic coast of the French Republic during the 1960s. The disease has spread to the Kingdom of Denmark, the Kingdom of the Netherlands, Ireland, the United Kingdom of Great Britain and Northern Ireland, the Italian Republic, the Kingdom of Spain and the west and east coasts of the United States of America. It seems that bonamiosis was originally introduced to the French Republic through the importation of oysters from California. Beside *O. edulis*, *O. lutaria* and *O. chilensis* are susceptible.

Recently, another *Bonamia* species, *B. exitiosa* was reported as a causative agent of disease in *O. edulis*.

Usually there are no typical clinical signs in affected oysters, however, sometimes it is possible to notice yellow discolouration or extensive lesions (even pustules) in the connective tissue of the gills, mantle and digestive gland. Prevalence of infection can vary from 10 to 90 percent, with low to high mortality rates in populations of *O. edulis*, especially when environmental conditions change or during periods of stress such as spawning.

The causative agent, *Bonamia ostreae*, is an intracellular plasmodial protistan parasite (2–5 µm in size) that affects the granular blood cells (haemocytes) of European flat oysters and causing pathology associated with haemocyte destruction and diapedesis due to proliferation of the parasite. Infection results in an increase in the number of tissue-infiltrating haemocytes.

Outbreaks may appear throughout the year but are more frequent in the warmer periods. In the natural habitat, it is possible to diagnose bonamiosis five months after introduction of healthy animals to the infected area, which demonstrates the horizontal transmission of the infection.

Diagnosis of infected oysters is made by histological or cytological examination and the use of molecular techniques. Histology can be carried out using standard histological procedures for molluscs. In histological slides, increased proliferation of haemocytes in the connective tissue, gill, gut or mantle epithelium can be observed, with *B. ostreae* inside the haemocytes and/or free in the connective tissue. Cytological examination can be carried out by observation of small parasites (2µm–5 µm) with basophilic cytoplasm and eosinophilic nucleii in heart imprints stained with Merck Hemacolour. Molecular techniques such as PCR may also be used to confirm the identification of *B. ostreae* in affected oysters. None of these methods can distinguish *B. ostreae* from *B. exitiosa*; therefore restriction fragment length polymorphism (RFLP) or gene sequencing should be performed to distinguish the species of *Bonamia*.

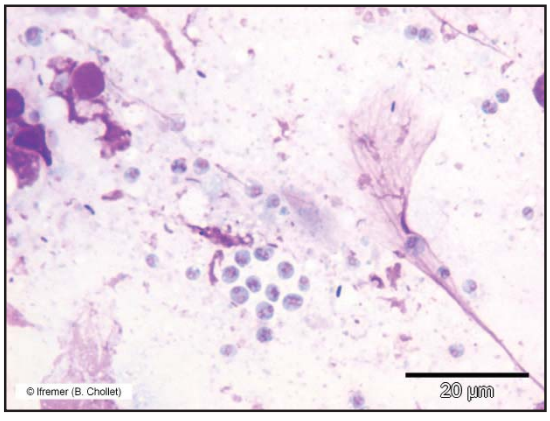

Figure 140. *Bonamia ostreae* inside a haemocyte. Impression smear stained with Hemacolour, Merck 100x (Photo courtesy of Bruno Chollet)

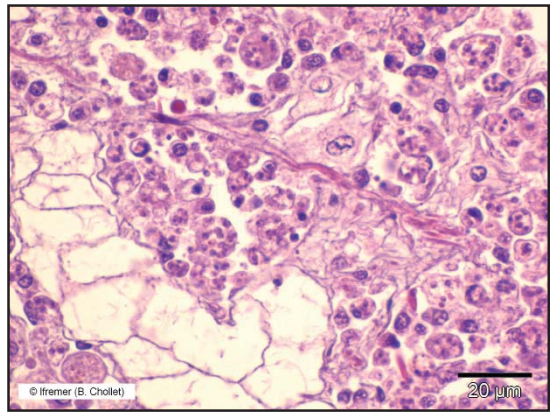

Figure 141. Haemocytes infected with *Bonamia ostreae*. Histological slide, H&E 100x (Photo courtesy of Bruno Chollet)

As in the case of marteiliosis, there is no possibility for eradication or treatment of the disease, and thus control should be based on import restrictions and a well-designed surveillance programme.

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ANNEX 1

PREPARATION OF MEDIA AND FIXATIVES

A. FIXATIVES FOR HISTOLOGY

Buffered neutral formalin (3.6%–4.0% formaldehyde)

Formalin (37%–40% formaldehyde)	100 ml
Distilled water	900 ml
Monosodium phosphate	18.6 g

pH should be approximately 7.0

Carson's fixative

Dissolve in 900 ml of distilled water:

Sodium didydrogenophosphate	23.8 g
Sodium hydroxide	5.2 g

Then add:

Formalin (36%–40%)	100 ml

Mix thoroughly

Davidson's fixative

Stock solution:

Filtered sea water	1 200 ml
Ethanol	1 200 ml
Formaldehyde (36%–40%)	800 ml
Glycerin	400 ml

Working solution:

Stock solution	9 parts
Glacial acetic acid	1 part *(add extemporaneously, i.e. just prior to use)*

B. MEDIA FOR BACTERIOLOGICAL DIAGNOSIS

Media preparation

The most of the commonly used bacteriological media like trypticase soy agar (TSA), blood agar (BA), marine agar (MA), brain heart infusion agar (BHIA), Mueller-Hinton agar (MHA) and thiosulfate-citrate-bile salts-sucrose agar (TCBS) are commercially available, and their preparation is according manufacturer's recommendation. Some specific media like kidney disease medium (KDM-2), selective kidney disease medium (SKDM) or *Cytophaga* and *Flexibacter maritimum* medium (FMM) agar should be prepared in the laboratory.

Plate Media

1. Prepare medium in stainless steel beakers or clean glassware according to manufacturer's instructions. Check pH and adjust if necessary. Medium must be boiled for one minute to completely dissolve agar.
2. Cover beaker with foil or pour into clean bottles, being sure to leave lids loose. Sterilize according to manufacturer's instructions (when given) or at 121°C for 15 minutes at 15 pounds pressure.
3. Cool medium to 50°C.

4. Alternatively, medium can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with medium type, date and initials. When medium is needed, boil, microwave or use a water bath to completely melt the agar. Cool to 50°C, then proceed to step 5.
5. Before pouring medium, disinfect hood or counter thoroughly and place sterile Petri dishes on the disinfected surface.
6. Label the plates or a plate storage tin with the type of medium, preparer's initials and date made.
7. Remove bottle cap and pour plates or dispense approximately 15 to 20 ml per 100×15 mm Petri dish. Replace lids as soon as the plate is poured.
8. Invert plates when the medium has cooled completely (~ 30–60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid. Do not use the ultraviolet (UV) light because it can denature the proteins in the medium.
9. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.
10. Follow manufacturer's recommendation for storage period of prepared medium.

Tube Media

1. Prepare medium in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely dissolve the agar.
2. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise compensation for temperature needs to be made.
3. Arrange test tubes in racks. Disposable screw-cap tubes can be used for all tube media.
4. Use an automatic pipettor or Pipet-aid™ to dispense the medium. Dispense approximately 5 to 10 ml medium in 16×125 mm or 20×125 mm tubes. Close caps loosely.
5. Follow manufacturer's recommendation for autoclave time and temperature.
6. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. short butt and long "fish-tail" slant for TSA or a standard slant over ¾ of the tube length for BHIA).
7. Tighten caps when tubes can be easily handled but still warm to the touch. Cool completely to room temperature in the slanted position.
8. Label the tubes or the tube rack with type of medium, preparer's initials and date made.
9. Store at 2°C–8°C, following manufacturer's recommendation for period of long-term storage.

Kidney disease medium (KDM-2)

Add measured amounts to a 2-litre flask:

Pepton	10.0 g
Yeast extract	0.5 g
Cystein-HCL	1.0 g
Agar	1.0 g
Deionized water	900 ml

Mix with stir bar and adjust pH to 6.5. Autoclave at 121°C for 15 minutes, cool to 50°C and add 100 ml FBS (10%). Store at 4°C in refrigerator.

Selective kidney disease medium

Add measured amounts to a 2-litre flask:

Peptone	10.0 g
Yeast extract	0.5 g
Cystein-HCL	0.5 g
Deionized water	900 ml

Mix with stir bar and adjust pH to 6.5 and add 10.0 g agar. Autoclave at 121°C for 15 minutes, cool to 50°C, add 100 ml FBS and antibiotics (cyclohexamide 0.05 g; D-cycloserine 12.5 mg; oxolinic acid 2.5 mg; polymyxin-B sulphate 15.0 mg). All antibiotics should be dissolved in distilled water, sterilized by filtration and added to the medium after autoclaving. Pour into plates and store at 4°C in refrigerator.

Cytophaga agar

For 1 liter of medium:

Trypton	0.5 g
Beef extract	0.2 g
Yeast extract	0.5 g
Na-acetate	0.2 g
Agar	9.0 g

FMM ("*Flexibacter maritimum* medium")

Peptone	5 g
Beef extract	0.5 g
Na-acetate	0.01 g
Agar	15.0 g

Sea water to 1 litre; pH 7.2 to 7.4

C. MEDIA FOR DIAGNOSIS OF VIRAL DISEASES

PBS (Phosphate buffered saline)

$NaH_2PO_4.H_2O$	1.726 g
$Na_2HPO_4.12H_2O$	13.4 g
NaCl	42.38 g
Milli Q water	1 litre

Dissolve all ingredients, and dilute with Milli Q water 1:5. pH should be 7.2±0.2.

Transport medium

EMEM	500 ml
FBS	50 ml
HEPES	11.25 ml
Antibiotic antimycotic mixture	5 ml

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ANNEX 2

ANTIMICROBIAL DRUGS APPROVED FOR TREATMENT OF FISH IN SOME COUNTRIES OF THE WESTERN BALKAN REGION

Bosnia and Herzegovina

Veterinary medicinal product	Active substance	Producer	Indication	Dosage	Withdrawal period
Trimetosul	Trimethoprim/Sulphafurazol	Genera, HR	Gram-negative bacterial infection	50 mg per kg body weight, daily in feed	350° days
Flumekvin-P 10%	Flumequine	"VETMEDIC" d.o.o the Republic of Serbia	Gram-negative bacterial infection	12–20 mg per kg body weight, daily in feed	80° days
OTC-VP 40%	Oxatetracycline	VETMEDIC" d.o.o the Republic of Serbia	Gram-negative bacterial infection	80 mg per kg body weight, daily in feed	720° days
Flumax 2%	Flumequine	Fish corp 2000, d.o.o., the Republic of Serbia	Gram-negative bacterial infection	10 mg per kg body weight, daily in feed	150° days
Flumekvin 10%	Flumequine	Veterinarski zavod Subotica, the Republic of Serbia	Gram-negative bacterial infection	12–20 mg per kg body weight, daily in feed	80° days
Egocin 20%	Oxatetracycline	Krka d.d., The Repubblic of Slovenia	Gram-negative bacterial infection	60 –80 mg per kg body weight, daily in feed	10°C; 90 days 20°C; 70 days 20°C; 60 days 6°C; 90 days
Floron 2%	Florfenicol	Krka d.d., The Repubblic of Slovenia	Gram-negative bacterial infection	10 mg per kg body weight, daily in feed	150° days

The former Yugoslav Republic of Macedonia

Veterinary medicinal product	Active substance	Producer	Indication	Dosage	Withdrawal period
Trimetosul	Trimethoprim/ Sulphafurazol	Genera, HR	Gram-negative bacterial infection	50 mg per kg body weight daily in feed	350° days
OTC-VP-40%	Oxytetracycline	VETMEDIC d.o.o the Republic of Serbia	Gram-negative bacterial infection	80 mg per kg body weight daily in feed	720° days
Flumekvin 10%	Flumequine	Veterinarski zavod Subotica, the Republic of Serbia	Gram-negative bacterial infection	12–20 mg per kg body weight daily in feed	80° days
Florfenikol-P 4%	Florfenicol	VETMEDIC d.o.o the Republic of Serbia	Gram-negative bacterial infection	10 mg per kg body weight daily in feed	150° days

The Republic of Croatia

Veterinary medicinal product	Active substance	Producer	Indication	Dosage	Withdrawal period
Geomycin 20%	Oxytetracycline	Genera, HR	Gram-negative bacterial infection	60–80 mg per kg body weight daily in feed	400° days
Egocin 20%	Oxytetracycline	Krka, SLO	Gram-negative bacterial infection	60 –80 mg per kg body weight daily in feed	10°C; 90 days 10–20°C; 70 days 20°C; 60 days 6°C; 90 days
Erythromycin 20%	Erythromycin	Genera, HR	Gram-positive bacterial infection	100 mg per kg body weight daily in feed	700° days
Trimetosul	Trimethoprim/ Sulphafurazol	Genera, HR	Gram-negative bacterial infection	50 mg per kg body weight daily in feed	350° days
Flumesyva 10%	Flumequine	Syva Laboratorios, the Kingdom of Spain	Gram-negative bacterial infection	12–50 mg per kg body weight daily in feed	80° days
Floron	Florfenicol	Krka, SLO	Gram-negative bacterial infection	10–30 mg per kg body weight daily in feed	80° days
Aquavac Vibrio		MSD Animal Health, Unied Kingdom	Vaccination against vibriosis by immersion		
Aquavac Vibrio Oral		MSD Animal Health, United Kingdom of Great Britain and Northern Ireland	Vaccination against vibriosis in feed		

The Republic of Serbia

Veterinary medicinal product	Active substance	Producer	Indication	Dosage	Withdrawal period
Florfenikol-P 4%	Florfenicol	VETMEDIC d.o.o the Republic of Serbia	Gram-negative bacterial infection	10 mg per kg body weight daily in feed	150^{o} days
Flumekvin-P 10%	Flumequine	VETMEDIC d.o.o the Republic of Serbia	Gram-negative bacterial infection	12–20 mg per kg body weight daily in feed	80^{o} days
OTC-VP 40%	Oxatetra-cycline	VETMEDIC d.o.o the Republic of Serbia	Gram-negative bacterial infection	80 mg per kg body weight daily in feed	720^{o} days
Flumax 2%	Flumequine	Fish corp 2000, d.o.o., the Republic of Serbia	Gram-negative bacterial infection	10 mg per kg body weight daily in feed	150^{o} days
Flumekvin 10%	Flumequine	Veterinarski zavod Subotica, the Republic of Serbia	Gram-negative bacterial infection	12–20 mg per kg body weight daily in feed	80^{o} days
Floron 2%	Florfenicol	Krka d.d., The Repubblic of Slovenia	Gram-negative bacterial infection	10 mg per kg body weight daily in feed	150^{o} days

ANNEX 3

List of Figures

2. Sampling of fish for diagnostic purposes

2.2 Clinical inspection and sampling on a salmonid farm

2.3 Clinical inspection and sampling of a cyprinid farm

2.4 Clinical inspection and sampling on the marine fish farm

2.5 Clinical inspection and sampling on the mollusc farm

3. Preservation, packing and shipping of samples

3.1 Shipping of live fish

3.2 Shipping of fresh fish

Figure 21. Fish are packed in a plastic bag and frozen plastic bottles or cooling blocks are used to maintain low temperature during transport. **(S. Zrnčić)**

3.3 Shipping of molluscs

Figure 22. Waterproof boxes for shipping mollucs. **(S. Zrnčić**)

Figure. 23. A written record is placed in a plastic envelope that is attached to the box cover. **(S. Zrnčić)**

3.4 Shipping of organs in transport media

Figure 24. Organs to be sampled for virological examination on a salmonid farm. **(S. Zrnčić)**

Figure 25. Organs to be sampled for virological examination on a carp farm. **(S. Zrnčić)**

Figure 26. Organs from ten fish could be placed into one tube with transport medium. **(S. Zrnčić)**

Figure 27. Volume of sampled organs should not exceed one third of the medium. **(S. Zrnčić)**

Figure 28. Organs in transport medium in a styrofoam box with freezing block. **(S. Zrnčić)**

Figure 29. Written data should accompany the shipment. **(S. Zrnčić)**

3.5 Shipping of material for histological examination

Figure 30. Sealed container containing tissues intended for histological analysis. **(S. Zrnčić)**

4. Diagnostic techniques

4.2.3 Examination of fresh organ preparation

Figure 31. Equipment needed for necropsy and sampling. **(S. Zrnčić)**

Figure 32. Fish sample prepared to be analyzed. **(S. Zrnčić)**

Figure 33. Presence of an isopod ectoparasite (*Ceratothoe oestroides*) in the mouth of European seabass. **(S. Zrnčić)**

Figure 34. Crustacean ectoparasite (*Lernanthropus kroyeri*) on the gill arch of European seabass. **(B. Šoštarić)**

Figure 35. Protozoan infection (*Ichthyophthirius multifiliis*) on carp gills. **(S. Zrnčić)**

Figure 36. Gill fluke (*Dactylogyrus* sp.) **(S. Zrnčić)**

Figure 37. Whitish nodules in enlarged spleen (pasteurellosis caused by *Photobacterium damselae* subsp. *piscicida* in seabream) and steatosis in the liver. **(S. Zrnčić)**

Figure 38. Haemorrhages on the pyloric caeca, peritoneum and intestine (viral hemorrhagic septicaemia in rainbow trout). **(S. Zrnčić)**

Figure 39. Haemorrhages on the gills and liver indicating bacterial infection (vibriosis caused by *Vibrio anguillarum* in European seabass). **(S. Zrnčić)**

Figure 40. Presence of endoparasites (*Echinorhynchus truttae* and *Cyathocephalus truncatus*) in the intestine of brown trout *(Salmo trutta* m. *fario*). **(S. Zrnčić)**

Figure 41. Scraping of seabream intestine infected with pansporoblasts and spores of the myxozoan *Enteromyxum leei.* **(S. Zrnčić)**

Figure 42. Stage of myxosporean (*Sphaerospora renicola*), the causative agent of swimbladder inflamation of carp (stained by Hemacolour kit). **(S. Zrnčić)**

4.2.4 Mollusc processing for diagnostics

Figure 43. Opening of mussels. **(S. Zrnčić)**

Figure 44. Oysters prepared for sampling of organs for histological examination. (the area between the red lines indicates the tissue to be taken). **(S. Zrnčić)**

Figure 45. Organization of organs in bay mussel. **(S. Zrnčić)**

Figure 46. Organization of organs in the European flat oyster. **(S. Zrnčić)**

4.3 Bacteriological techniques

4.3.2 Identification of pathogenic bacteria

Figure 47. Different bacteriological media for the isolation of fish bacteria. **(S. Zrnčić)**

Figure 48. Restreaking of bacterial colonies to achieve pure colony. **(S. Zrnčić)**

Figure 49. Smear of bacterial colony stained by Gram staining. **(S. Zrnčić)**

Figure 50. Presence of cytochrome oxidase C in isolated bacteria. **(S. Zrnčić)**

Figure 51. BBL™ Crystal™ Enteric/ Nonfermenter ID Kit for identification of bacteria. **(S. Zrnčić)**

Figure 52. API 20E kit for identification of Gram-negative bacteria. **(S. Zrnčić)**

4.3.2 Determination of sensitivity to antimicrobials

Figure 53. Susceptibility of isolated bacteria to antimicrobials (disc diffusion method). **(S. Zrnčić)**

Figure 54. Susceptibility of isolated bacteria to antimicrobials (minimum inhibitory concentrations). **(S. Zrnčić)**

4.4 Virological techniques

4.4.2 Sampling and processing of organs for virological examination on cell cultures

Figure 55. Homogenization of sampled tissue and dilution with “transport medium”. **(S. Zrnčić)**

Figure 56. Homogenized and diluted material. **(S. Zrnčić)**

Figure 57. Material should be centrifuged in a cooling centrifuge (4 ^{o}C)for 20 minutes at 5 000 rpm. **(S. Zrnčić)**

Figure 58. Centrifuged material for virological examination. **(S. Zrnčić)**

Figure 59. Filtration through 0.45 µm pore to avoid bacterial contamination. **(S. Zrnčić)**

Figure 60. Material ready for virological inoculation on to cell cultures. **(S. Zrnčić)**

4.4.4 Virus isolation on the cell cultures

Figure 61. Incubator with different cell cultures for virus isolation. **(S. Zrnčić)**

Figure 62. All operations should be performed in a sterile laminar flow unit. **(S. Zrnčić)**

Figure 63. Bottle with trypsinized cell culture. **(S. Zrnčić)**

Figure 64. 96-well plate with EPC and BF2 cell lines. **(S. Zrnčić)**

Figure 65. EPC healthy cell line. **(S. Zrnčić)**

Figure 66. CPE caused by virus on EPC cell line. **(S. Zrnčić)**

4.4.5.2 ELISA (enzyme-linked immunosorbent assay)

Figure 67. Wells in microtiter plates for detection of different viruses. **(S. Zrnčić)**

Figure 68. ELISA reader. **(S. Zrnčić)**

4.5 Histology

4.5.1 Tissue fixation and processing for histological purposes

Figure 69. Tissue placed into the plastic cassette. **(S. Zrnčić)**

Figure 70. Cassettes with tissues are fixed in formalin for at least 24 hours. **(S. Zrnčić)**

Figure 71. Dehydration of tissues in a tissue processor. **(S. Zrnčić)**

Figure 72. Embedding of processed tissue in paraffin. **(S. Zrnčić)**

Figure 73. Tissues embedded in paraffin blocks, ready to be cut. **(S. Zrnčić)**

Figure 74. Histo-slide with hematoxylin-eosin (H&E) stained tissue mounted with a coverslip. **(S. Zrnčić)**

4.5.2 Staining techniques

Figure 75. Photomicrograph of carp liver tissue with several islands of pancreatic tissue (hematoxylin and eosin staining). **(B. Šoštarić)**

Figure 76. Opisthaptor of the monogenean *Diplectanum aquans* embedded in desquamated gill epithelium of the European seabass (Toulidine blue). **(I. Mladineo)**

Figure 77. Xenoma-forming *Microsporidium* sp. in connective tissue of *Knipowitschia croatica*. (Masson's trichrome). (**I. Mladineo)**

4.6 Molecular methods

Figure 78. DNA extraction from tissue of pure bacterial or viral culture. **(V. Radosavljević)**

Figure 79. Thermo-cycler. **(V. Radosavljević)**

Figure 80. Visualization of DNA in agarose gel. **(V. Radosavljević)**

Figure 81. Curve showing cycle threshold (ct). **(S. Zrnčić)**

Figure 82. Sequencing as a substrate for phylogenetic and epidemiological research. **(S. Zrnčić)**

Figure 83. *In situ* hybridization visualizes much more pathogen compared to histology (*Martiela refringens* in the digestive gland of mussel). **(S. Zrnčić)**

5. Pathogens of regional importance

5.1 Viral diseases5.2 Viral haemorrhagic septicaemia (VHS)

Figure 84. Haemorrhages on the fin bases and in the eyes are typical clinical signs. **(S. Zrnčić)**

Figure 85. Uni- or bilateral exophthalmia is often visible in infected fish. **(S. Zrnčić)**

Figure 86. Distended abdominal wall due to ascites, and haemorrhages on the skin. **(S. Zrnčić)**

Figure 87. Extensive haemorrhages on the swimbladder, pyloric caeca, intestine and peritoneum. **(S. Zrnčić)**

Figure 88. Anaemia, pale liver with haemorrhages, haemorrhages on the intestine and in the dorsal muscle. **(S. Zrnčić)**

Figure 89. Phylogenetic analysis places Croatian isolate into genotype Ia, subtype 2, subclade Pol II. **(S. Zrnčić)**

5.3 Infectious haemopoietic necrosis (IHN)

Figure 90. Dark pigmentation; haemorrhages on the fat tissue, in the musculature and peritoneum. **(S. Zrnčić)**

Figure 91. Haemorrhages on the swimbladder, intestine, pyloric caeca and fat tissue. **(S. Zrnčić)**

Figure 92. Anaemia, haemorrhages in the gills. **(S. Zrnčić)**

Figure 93. Splenomegaly. **(S. Zrnčić)**

Figure 94. Dark pigmentation and exophthalmia are usually the first clinical signs in the infected facility**. (V. Jenčič)**

Figure 95. Phylogenetic analysis of Croatian isolate of IHNV. **(S. Zrnčić)**

5.4 Koi herpesvirus disease (KHVD)

Figure 96. Pale, irregular patches on the skin associated with excess mucus secretion, superficial haemorrhaging**. (A. Sunarto)**

Figure 97. Sunken eyes with haemorrhages, pale necrotic patches to extensive discolouration, severe necrosis and inflammation of the gills. **(V. Radosavljević)**

Figure 98. Severe gill necrosis and inflamation of the gills of koi carp. **(O. Haenen)**

Figure 99. Histo-section of carp gill infected with KHV. Note vacuolization and intracellular inclusions in the infected cells (arrows). **(M. Satoshi)**

5.5 Spring viraemia Of carp (SVC)

Figure 100. Haemorrhages in the kidney and swimbladder and pale liver in carp infected by SVC. **(V. Radosavljević)**

Figure 101. Cytopathic effect (CPE) in EPC cell lines caused by *Rhabdovirus carpio.* **(V. Radosavljević)**

5.6 Infectious pancreatic necrosis (IPN)

Figure 102. Rainbow trout fry infected with IPNV (note dark pigmentation, distended abdomen). **(V. Radosavljević)**

Figure 103. Necrotic changes in pancreas of IPN-positive rainbow trout fry. **(I. Mladineo)**

5.7 Viral encephalopathy and retinopathy (VER)

Figure 104. Abnormal swimming behaviour of European seabass fry (spiral swimming). **(S. Zrnčić)**

Figure 105. Affected specimens are thin, with lesions on the head and mouth. **(S. Zrnčić)**

Figure 106. Corneal opacity is often visible in affected specimens. **(S. Zrnčić)**

Figure 107. Lesions and haemorrhages on the mouth and head. **(S. Zrnčić)**

Figure 108. Congestion and haemorrhages on the brain are visible on necropsy. **(S. Zrnčić)**

Figure 109. Vacuolization in the retina of fish infected by Betanoda virus (H&E).

6. Bacterial disease

6.1 Bacterial kidney disease (BKD)

Figure 110. Infected rainbow trout exhibiting haemorrhages on the liver, granulomatous lesions on the kidney, and splenomegalia. **(V. Radosavljević)**

Figure 111. Granulomatous lesions in the liver and kidney of rainbow trout **(D. Oraić)**

6.2 Yersiniosis (enteric redmouth disease – ERM)

Figure 112. Petechial haemorrhages on the liver and pyloric caeca, and splenomegaly in infected rainbow trout. **(V. Radosavljević)**

Figure 113. Haemorrhages around and in the mouth. **(D. Oraić)**

Figure 114. Pure bacterial colonies on blood agar. **(V. Radosavljević)**

Figure 115. Short, Gram-negative rods, *Yersinia ruckeri.* **(V. Radosavljević)**

6.3 *Aeromonas* infections

Figure 116. Carp infected with MAS. **(V. Radosavljević)**

Figure 117. Colonies of *Aeromonas hydrophila* isolated from diseased carp. **(V. Radosavljević)**

Figure 118. Lesions on the body trunk of common carp typical for CE. **(S. Zrnčić)**

Figure 119. Healed ulcer forming a scar. **(S. Zrnčić)**

6.4 Vibriosis

Figure. 120. Haemorrhages on the head, opercula and the fin bases in the acute form of vibriosis. **(S. Zrnčić)**

Figure 121. Subacute form of vibriosis with more pronounced haemorrhages on the mouth, head, fin bases, fins and abdominal wall. **(S. Zrnčić)**

Figure 122. Chronic form of vibriosis characterized by skin ulceration. **(S. Zrnčić)**

Figure 123. Necropsy reveals haemorrhages on the liver, stomach and intestine (subacute to chronic course). **(S. Zrnčić)**

Figure 124. Growth of *Vibrio anguillarum* (yellow colonies) on selective, TCBS agar. **(S. Zrnčić)**

Figure 125. Revaccination of European seabass fry against *Vibrio anguillarum* by immersion. **(S. Zrnčić)**

6.5 Infections with “yellow-pigmented bacteria”

Figure 126. Chronic course of bacterial coldwater (BCW) disease in brook trout with gill destruction. **(D. Oraić)**

Figure 127. Rainbow trout fry syndrome (RTFS) in rainbow trout fry (note ascites, enlarged spleen). **(S. Zrnčić)**

Figure 128. Lesion on the external surface of rainbow trout due to infection with *Flavobacterium psychrophila.* **(B. Šoštarić)**

Figure 129. "Column-like" lesion on the skin of common carp infected with *Flavobacterium columnare* **(D. Oraić)**

Figure 130. Infected fish are cachectic, with visible scale loss and skin lesions. **(S. Zrnčić)**

Figure 131. Pronounced skin lesions on the head. **(S. Zrnčić)**

Figure 132. Swollen, anaemic gills. **(S. Zrnčić)**

Figure 133. Generalized anaemia, intestine filled with liquid content and fibrinous cast. **(S. Zrnčić)**

Figure 134. Long Gram-negative rods, *Tenacibaculum maritimum.* **(S. Zrnčić)**

Figure 135. PCR diagnosis of *Tenacibaculum maritimum.* **(S. Zrnčić)**

7. Epizootic ulcerative syndrome (EUS)

Figure 136. *Aphanomyces invadans* (= *A. piscicida*) infection in Japanese pond-cultured ayu, *Plecoglossus altivelis* **(Prof. K. Hatai)**

Figure 137. Histopathology of EUS-suspected barb showing: (i) typical mycotic granulomas surrounding the invasive fungal hyphae penetrating into the muscle layer (H&E stain) (left photo); and (ii) typical mycotic granulomas surrounding the invasive fungal hyphae in the skin layer (Grocott's stain) (right photo). (FAO, 2009).

8. Diseases of molluscs

Figure 138. *Martiela refringens* in the imprint of the digestive gland of mussel (*Mytilus galloprovincialis*) stained with Hemacolour kit 40x **(S. Zrnčić)**

Figure 139. *Martiela refringens* parasiting the digestive gland tubuli of *Mytilus galloprovincialis*. Histological section, H&E, 40x **(S. Zrnčić)**

Figure 140. *Bonamia ostreae* inside a haemocyte. Impression smear stained with Hemacolour, Merck 100x **(B. Chollet)**

Figure 141. Haemocytes infected with *Bonamia ostreae*. Histological slide, H&E 100x **(B. Chollet)**

ANNEX 4

Photo documentation of the Regional Workshop 2: Improving Capacity for Diagnosis of Diseases of Fish and Molluscs Banja Luka, Bosnia and Herzegovina, 20–24 October 2013

Figure 1. Group photo of Workshop participants

Figure 2. Opening statements by Dr. Pavo Radić, Deputy Director of MoFTER, BIH and Dr. Sanin Tanković, TCDC/ TCCT No 1

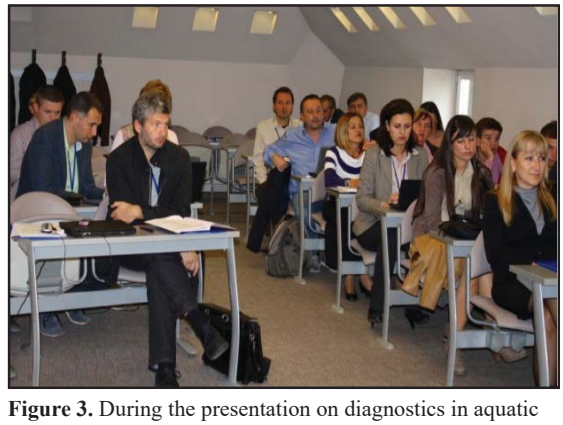


Figure 3. During the presentation on diagnostics in aquatic animal health

Figure 4. Consultants are resuming last session and discussing about following

Figure 5. Working group discussion on possibilities, needs and capacity for NRL in particular Western Balkan Countries

Figure 6. Working group discussion on scope and contents of regional diagnostic manual

Figure 7. Group photo of participants on the field trip

Figure 8. Farm manager providing an introduction to rainbow trout to the participants

Figure 9. Person feeding the fish is the first one to notice disease signs

Figure 10. Sick fish tend to aggregate in the water inlet area

Figure 11. Clinical inspection of the fish tanks was demonstrated to the participants

Figure 12. Demonstration on how to collect the most appropriate specimens for disease diagnostics

Figure 13. Organs of rainbow trout should be collected into transport media

Figure 14. Participants were instructed how to dissect rainbow trout

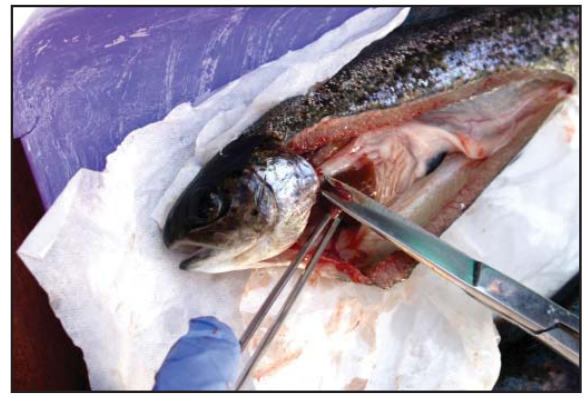


Figure 15. Targeted organs for presence of listed diseases were selected

Figure 16. & Figure 17. Each participant was trained to sample organs for viral diseases diagnostics

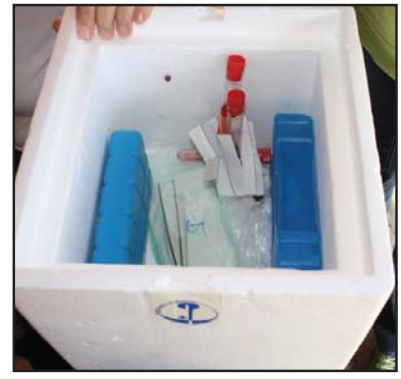
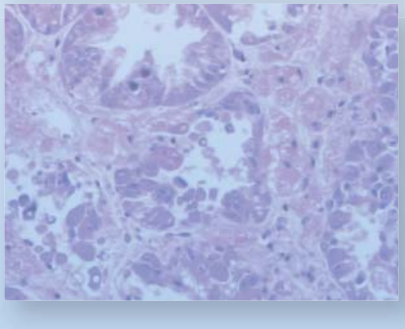
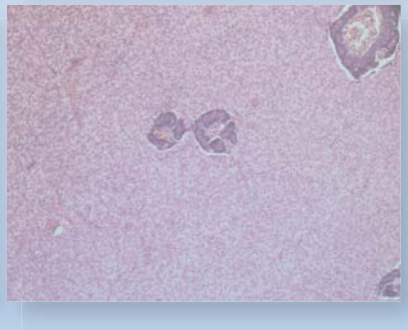


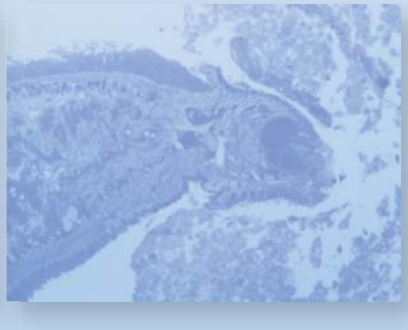
Figure 18. Packing and shipping methods were demonstrated on the spot

Figure 19. Field trip finalized pleasantly due to hospitable fish farm stuff

Figure 20. Group photo during the visit to the regional veterinary diagnostic institution







TCP/RER/3402

Assistance to Western Balkan Countries for Improving Compliance to International Standards on Aquatic Animal Health

The West Balkans Regional Aquatic Animal Disease Diagnostic Manual is a comprehensive handbook for official veterinarians, veterinary inspectors and fish health experts working at the aquaculture farms in five Western Balkan countries. It is developed through the Food and Agriculture Organization of the United Nations (FAO) Technical Cooperation Programme Project TCP/RER/3402 “Assistance to Western Balkan Countries for Improving Compliance to International Standards on Aquatic Animal Health”, a regional TPC project based on a consultative and consensus-building process. This is a diagnostic guide for surveillance, clinical inspections and sampling at the aquaculture facilities with the aim of meeting the standards of the World Organisation for Animal Health (OIE), as set out in the *Aquatic Animal Health Code and the Manual of Diagnostic Tests for Aquatic Animals*, and the European Union's (EU) Council Directive 2006/88/EC of 24 October 2006 on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals, for diseases listed by these standards, as well as other diseases of economic importance. Both standards include monitoring for diseases and the obligatory notification of clinical signs in registered farms. It provides knowledge on diseases, farm production, normal appearance of the farmed species, and recognition of any changes. It gives detailed instructions for clinical inspections of the fish or mollusc farms, recognition of unusual behaviour, selection of appropriate specimens for laboratory examination; collection, packing and shipping samples to the diagnostic laboratory, description of laboratory procedures for detection of disease agents as well as information on viral and bacterial finfish diseases and the diseases of molluscs.

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