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Marine Environment and Resources Erasmus Mundus PhD Program

Towards Intelligent Aquaculture - Development of an early Biological Warning System to monitor exposure to contaminants and fish welfare: from artificial vision to systems modelling

International PhD Thesis submitted by

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For the degree of

Philosophiae Doctor

Under the supervision of Prof Iciar Martinez and Dr Karmele Lopez de Ipiña.

Plentzia, December 2015

For Edur,
for Berta,
and for Aitite Cesar (*in memoriam*).

Image from http://www.ehu.eus/PIE

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PREFACE

This Thesis is submitted in partial fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the University of the Basque Country UPV/EHU under the Marine Environmental and Resources Erasmus Mundus PhD program.

The work performed in the present document was carried out at the Research Center for Experimental Marine Biology and Biotechnology - Plentzia Marine Station (PiE) of the University of the Basque Country UPV/EHU from February 2013 to December 2015. During that period, a research stay was carried out from April to July 2015 at the Centre for Autonomous Marine Operations and Systems (AMOS) of the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway.

In order to allow a better reading and understanding of the text, acronyms have been left to the minimum. The present document is organized into Chapters. Additionally and from a conceptual point of view, it can be divided into four Parts. A brief description of them follows:

- Part I provides the context to this Thesis and it is composed of the first two chapters.
    - Chapter 1 covers the general introduction of this work. It also gives a brief view of the composition of the entire document: it includes the Hypothesis, the Objectives and Contributions, and the Scientific Output derived from the work.
    - Chapter 2 is devoted to the Research Methodology used. The research plan followed during the Thesis and the benefits derived from it are presented. Specific Research Methods and Limitations of the present study are also addressed.
- Part II describes the development of the Biological Warning System from its early stage to the inclusion in a mathematical model. It comprises the next five chapters.
    - Chapter 3 is a brief explanation of State of the Art in Biological Warning Systems.
    - Chapter 4 deals with the development of the monitoring tool.
    - Chapter 5 quantifies the effect of the variation in the number of fish on the system dynamics.
    - Chapter 6 presents a particular case of study of the monitor tool applied to a contamination exposure example.
    - Chapter 7 merges the knowledge generated in the previous chapters and builds a mathematical model of the system.
- Part III presents the General discussion, Conclusions and the Thesis. It also establishes the future prospects. This part constitutes Chapter 8.

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Made weak by time and fate, but strong in will
To strive, to seek, to find, and not to yield.

Ulysses. Lord Alfred Tennyson

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ABSTRACT

The annual global increase in the production, particularly from aquaculture, and consumption of seafood is expected to continue in the future. One of the main concerns is the production of safe seafood, which is being compromised by the increasing number of novel and unexpected chemical substances that contaminate the aquatic environment and may end up contaminating also the feeds used in aquaculture. Many of these contaminants also affect negatively the fish welfare and consequently carry economic losses for the farmer.

Currently, there is a lack of cost effective, user-friendly methods to detect many of these contaminants and there is no method to detect unknown contaminants. The present Thesis aims at developing a non-invasive monitoring methodology using the fish themselves as the sensor unit; i.e. a Biological Warning System. This kind of approach would make it possible to develop an affordable, on-line identification of production units displaying atypical responses or behaviour due to stress factors- and therefore potentially contaminated - regardless of whether the contaminant is an identified or an unknown substance. Once developed, the Biological Warning System should be implemented within the Hazard Analysis and Critical Control Point and Fish Welfare Assurance Systems and the results accompany the traceability documentation of the products.

The first phase of this Thesis comprises the development of a non-invasive methodology for image acquisition, image processing and non-linear signal analysis and processing of the collective fish response to a stochastic event, using the collective fish response, i.e., the response of the shoal. Object detection and motion estimation were performed by an optical flow algorithm in order to detect moving fish and simultaneously eliminate background, noise and artifacts. The entropy and the fractal dimension of the trajectory followed by the centroids of the groups of fish were calculated using the Shannon and permutation entropy and the Katz, Higuchi and Katz-Castiglioni's fractal dimension algorithms respectively.

The tool developed was tested in three case studies. The first case study analyzed three experimental groups of European seabass (*Dicentrarchus labrax*), two of which were similar (control and tagged fish) and very different from the third [tagged fish submerged in methylmercury (4 μg MeHg/L) contaminated water for 9 days]. The results indicate that Shannon entropy and Katz-Castiglioni were the most sensitive non-linear signal processing algorithms, both with the potential to become useful tools for the non-invasive identification and quantification of differences in fish responses, although the Katz-Castiglioni fractal algorithm had a much higher computational load than the Shannon entropy algorithm.

The number of individuals in the shoal was, a priori, expected to exert an influence in the behaviour of the system. Accordingly, the second case study was designed to identify the effect of the number of fish on the "normal" behaviour of the system, which in turn requires the characterization of the Optimal Working Point/Range

of the system. Conceptually, an optimal working point/range is the set of conditions under which the system operates in optimal and/or almost optimal conditions, i.e., where the system operates under what is considered to be acceptable conditions. In case something drives the system out from that point/range, the monitoring tool should be able to detect the deviation. For that purpose two different experiments where performed:

i. A decreasing biomass experiment, starting with 50 fish and decreasing to 25, 13 and finally 1 fish.

ii. An increasing biomass experiment, studying the system with initially 1 fish, adding 1 new fish per day during 5 days, and ending with 5 fish in the tank.

The third case study examined the response of European seabass system during the exposure to two different substances: sodium selenite (Na_2SeO_3, 10 µg/L) for 7 days and methylmercury (4 µg MeHg/L) for 14 days.

The results from the first case study indicate that the developed tool was suitable to identify variations in the response of a fish group to an event. The tool worked satisfactorily when applied to a complex, challenging, real-life experimental set up, and it rendered meaningful results that sustain the hypothesis of this Thesis.

The results of the second case study indicate that the Shannon entropy of the fish system was the most suitable parameter to analyse the trajectory of the cluster's centroid, and that its value was highly dependent on the number of European seabass for a few fish (from 1 to 5) becoming more independent from the number of individuals as their number increased. The relationship between the Shannon entropy of a European seabass system and the number of fish was shown to fit an exponential curve.

The results of the third case study indicated that the tool did not register alterations in Shannon entropy of the system upon the addition of sodium selenite in concentrations expected to exert a protective effect on to the European seabass health; but upon de addition of the neurotoxic methylmercury, in a concentration expected to exert a negative effect, the Shannon entropy of the system changed, producing lower values. Moreover, application of the tool showed that, not only the Shannon entropy value, but also its daily evolution need to be included as parameters in a fish welfare monitoring procedure for a European seabass system as shown by the case when the fish are recuperating from methylmercury exposure.

The last part of the Thesis deals with the development of a model, based on the work performed, that integrates the daily behaviour of three reference sub-models; namely, the basal state reference sub-model (corresponding to the resting fish), the response to the stochastic event reference sub-model (corresponding to the response of the fish system to a hit in the tank) and a sub-model based on the relationship between the two of them: the basal-event response reference sub-model.

Based on these results, it seems that the developed tool, after the necessary improvements and optimizations, has the potential to be embedded in an on-line/

real time architecture to monitor fish schools in a farm and in the wild, and therefore may find an application as a monitoring tool in Fish Welfare Assurance System and Hazard Analysis and Critical Control Points systems in fish farming, and to identify contaminated waters in environmental monitoring programs.

Accordingly, the Thesis of this study is that fish can be used as biological sensors because the alteration of their behaviour in response to external stimuli is quantifiable and can be non-invasively monitored. Further, the alteration of the behaviour as measured by the Shannon entropy of the system has the potential to serve as a tool for on-line fish welfare monitoring.

CHAPTER 1
INTRODUCTION

1.1. General Context

Gerland (2014) concluded, after analysing the world population projections published by the United Nations (2014), that: "...contrary to previous literature, world population is unlikely to stop growing this century. There is an 80% probability that world population, now 7.2 billion, will increase to between 9.6 and 12.3 billion in 2100". Thus, while predictions change depending on wars, pandemics and changes in demographics, it seems clear that there will be a need to increase and optimize the production of food for the human population. Aquaculture is considered to be one of the key production methods to achieve this goal (German Advisory Council on Global Change - WBGU, 2013) and some of the most recent international strategies consider the marine environment the last frontier to relaunch global economy (Kalogerakis et al., 2015). Seafood is the furthermost traded food worldwide and yet it is a highly ignored component in global food security context (Smith et al., 2010). The increasing relevance of seafood in the human diet is not new: according to FAO (2014), global fish production has been growing steadily during the last 50 years, with seafood supply increasing at an average annual rate of 3.2 % where most of the growth in seafood production in later years has been due to the dramatic increase in aquaculture production in China. The top in aquaculture production took place in 2012, with 90.4 million tonnes of aquatic seafood (66.6 million tonnes of fish and 23.8 of aquatic alga) for an estimated total value of US$144.4 billion. The estimates for 2013 were 70.5 and 26.1 million tonnes of fish and aquatic alga respectively. World food fish aquaculture production has increased at an average annual rate of 6.2% from 2000-2012, mostly in Africa, Latin America, the Caribbean and Asia (FAO, 2014).

On the 28th of January 2002 the European Parliament and the Council adopted Regulation (EC) 178/2002 laying down the General Principles and requirements of Food Law. The Regulation establishes the basic principle that the primary responsibility for ensuring compliance with food law, and in particular the safety of the food, rests with the food business. The same principle applies to feed production. The food business operators must therefore take all the necessary measures to ensure that the food they produce is fit for human consumption. The safety of the produced seafood remains therefore a highly relevant central issue, particularly in view of the increase in global trade, the different demands and expectations in different world regions and the number and variety of unexpected contaminants that have been recently discovered. Thus, to ensure the safe production of seafood, the implementation of the Hazard Analysis and Critical Control Point (HACCP) system and Risk Analysis concepts have become obligatory. FAO has published some very useful technical reports on the subject (Huss, 1994; Huss et al., 2004). Seafood can become contaminated at any step during its production and processing from the egg to the table (Huss et al., 2004). The main routes through which contaminants are introduced during fish production are the feed, the water and the veterinary treatments (Dahle et al., 2010). The type of contaminants one would expect to find vary according to factors such as whether the fish is farmed or wild, placed high or low in the trophic chain, its feed or prey, age and geographical origin, water temperature and proximity to populated areas, terrestrial

farms or industrial activities. Additionally, some contaminants, such as methyl-mercury, until recently considered to be of concern only in large, long-lived marine organisms placed high in the trophic chain (such as whales, swordfish and tunafish), have been found to be ubiquitous in the aquatic environment (Chen et al., 2012) and present in detectable amounts in a large variety of species and seafood products of wild and farmed fish species from different geographic origins including the Atlantic (Vieira et al., 2011), the Arctic, the Antarctic, Asia and the UK (Knowles et al., 2003), South China, Spain, USA and Australia (Qiu et al., 2011).

Farmed fish can be contaminated with agents that may induce infections (bacteria, viruses, parasites) and intoxications (tetrodotoxin, histamine, algal toxins, environmental pollutants, drugs and medicines, heavy metals, etc.) in humans. Some of these compounds can be toxic to humans and innocuous to the carrier agent, i.e., the fish. Tetrodotoxin for example, synthesized by symbiotic bacteria in some tissues of fish and snails, not only does not interfere with the life or welfare of the host: it improves both by acting as a protection against predators (Simidu et al., 1987). However, many other marine toxins, such as those caused by harmful algal blooms (HAB) exert a negative influence on the fish welfare and health, occasionally causing large mortalities (Bushaw-Newton and Sellner, 1999).

1.2. Fish welfare and fish welfare monitoring

Animal welfare refers to the well being of animals. It is based on the belief that they are sentient beings whose well being needs to be taken into consideration, particularly when they are under human care, as is the case of pets and animals used for the production of food and for research.

The concept of the Five Freedoms in animal welfare originated with the "Report of the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems"; known as the Brambell Report (1965), that stated that farm animals should have freedom "to stand up, lie down, turn around, groom themselves and stretch their limbs". This list is still nowadays referred to as Brambell's Five Freedoms.

As a direct result of the Brambell Report, the Farm Animal Welfare Advisory Committee (FAWAC) was set up and later replaced by the Farm Animal Welfare Council (FAWC) established by the British Government in July 1979. One of these bodies started to list the provisions that should be made for farm animals in five categories, which also became known as the Five Freedoms. Since records from FAWAC are not readily available the exact origin is not clear and the earliest written reference the author can find is a press notice (Figure 1.1) released by FAWC in December 1979.

FARM ANIMAL WELFARE COUNCIL

PRESS STATEMENT December 5, 1979

In his statements on 25th July and 4th December, the Minister of Agriculture, Fisheries and Food announced that the terms of reference of the Farm Animal Welfare Council would be to keep under review the welfare of farm animals on agricultural land, at markets, in transit and at the place of slaughter, and to advise the Agriculture Ministers of any legislative or other changes that may be necessary. The Council is free to publicise its views and will do so whenever, as now, circumstances make it appropriate. Ministers have asked the Council to advise as speedily as possible on revisions to the Welfare Codes for Cattle, Pigs, Domestic Fowls and Turkeys and to undertake this revision in such a way as to reflect advances in scientific knowledge and husbandry practice since the Brambell Committee reported in 1965. In preparing revisions of these Codes, the Council will build on the valuable work already done by the Farm Animal Welfare Advisory Committee, now disbanded.

The Codes are not mandatory but intended to create the best possible standards of welfare for animals in all systems of livestock husbandry, both intensive and extensive. The Council wishes the revised Codes to provide farm animals with the following:-

(1) freedom from thirst, hunger or malnutrition;
(2) appropriate comfort and shelter;
(3) prevention, or rapid diagnosis and treatment, of injury and disease;
(4) freedom to display most normal patterns of behaviour;
(5) freedom from fear.

The Codes are intended to contain more specific recommendations than formerly and should, in particular, place more emphasis on behavioural needs.

The Council may not necessarily be able to endorse every husbandry practice used on livestock farms but, until changes can be achieved, believes that it is in the interest of the animals to continue to give advice on the best possible management within those systems.

In addition to advising on revisions of the Codes, the Council will give careful consideration to the adequacy of farm animal welfare legislation in all the areas to which its remit extends, on the farm, in markets, during domestic transport, during export and at the place of slaughter.

Throughout its work the Council recognises the need for increased knowledge of the physiological and behavioural needs of farm animals, and, where research and development on this work appears to be insufficient, it will ask for work to be undertaken. Moreover, the Council also accepts that animal welfare raises certain points of ethics which are themselves beyond scientific investigation. The Council will therefore especially wish to encourage alternative systems of livestock husbandry which are ethically acceptable to the concerned public, can be shown to improve the welfare of the livestock in question and be economically competitive with existing systems of intensive production.

The Council will take a close interest in the work of the Standing Committee set up under the Council of Europe Convention for the Protection of Animals kept for Farming Purposes. Welfare developments in the E.E.C. are also a matter of interest to the Council which will keep itself informed so that it can offer advice to Ministers as appropriate. In this, and in all its other tasks, the Council is free to publicise its views and is accessible to any organisation or individuals.

Enquiries and correspondence should be addressed to the Secretary, Mr. R. Townsend, Farm Animal Welfare Council, Government Buildings, Hook Rise South, Tolworth, Surbiton, Surrey, KT5 7NF (01-337 6611, ext. 583).

Figure 1.1. 1979 press release from the Farm Animal Welfare Committee. From http://webarchive.nationalarchives.gov.uk/20121007104210/http://www.fawc.org.uk/pdf/fivefreedoms1979.pdf

Nowadays, the five freedoms of animal welfare are considered to be (Figure 1.2):

1. Freedom from hunger and thirst by ready access to fresh water and a diet to maintain full health and vigour.
2. Freedom from discomfort by providing an appropriate environment including shelter and a comfortable resting area.
3. Freedom from pain, injury or disease by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour (or as close as possible) by providing sufficient space, proper facilities and company of the animal's own kind.
5. Freedom from fear and distress by ensuring conditions and treatment, which avoid mental suffering.

Figure 1.2. The Five Freedoms in animal welfare. Image from: Microsoft Word 2011 for Mac.

The welfare of the animals and their health are closely interrelated: the presence of toxic substances and contaminants have a direct and negative impact on both, and factors that increase the stress of the animal - discomfort, lack of proper space, injury and disease and distress - will make them more susceptible to suffer injuries and disease (Broom, 1991). Broom (1991) proposed that the term "welfare" should be used by the scientific community and be included in laws. He also indicated the need to objectively measure welfare.

In general, welfare refers to the ability of an individual to cope, and central to all definitions is the association between the poor welfare of an individual and an overtaxing of its coping capacity. Responses to short-term stress are adaptative (to recuperate the temporarily lost homeostasis) and do not necessarily indicate suffering or diminished welfare. Concern about welfare in aquaculture is mainly associated with tertiary effects of stress response usually indicative of prolonged, repeated or unavoidable stress. Physiological indicators of poor welfare include: reduced life expectancy, impaired growth, impaired reproduction, body damage, disease, immunosuppression, adrenal activity, behaviour anomalies, and self-narcotization (Broom, 1991). The biology of fish welfare is indeed a complex matter that has been the subject of many studies, including two recent PhD Theses (Boerrigter, 2015; Manuel, 2015).

Factors with a negative impact on welfare also have a direct and negative impact on the quality and wholesomeness of the foodstuffs produced from them. Thus, it is very important to monitor and quantify the welfare of the animals under production to ensure that it is optimal. In support of this view, Dr Sunil Kadri, in his 2015 presentation to the European Aquaculture Society Meeting in Rotterdam, Amsterdam ("Fish Welfare: moving towards animal-based indicators" Kadri, S. and Keeling, L.) indicated that good fish welfare improves the health of the animals and the quality of the product. For the farmer this means a higher income and reduced production costs. The higher income would not only be due to a potentially higher willingness to pay from some customers, but also to the fact that the fish quality itself would be higher (Sneddon, 2007) and that fewer fish will die under production. Dr Kadri reported that 38% of the usually recorded 20% mortality during the on-growing period of salmon in Norway, could be directly attributed to poor handling and that improved welfare monitoring and control could reduce this figure.

The needs to define the concept of fish welfare in aquaculture and the development methods of objectively measure it were reviewed by Ashley (2007) who indicated that in addition to the already established physiological parameters that indicate suboptimal welfare (a classical example is the cortisol levels), also the much less studied effects that poor welfare may have on fish behaviour should be taken into account, stressing the need to obtain more knowledge about the behavioural responses of fish, how to measure them and how to relate them to fish welfare.

Dr. van de Vis, one of the European pioneers in the study fish welfare, and his co-workers proposed to follow the same principles that the Hazard Analysis and Critical Control Points (HACCP) establishes to safeguard food safety, but oriented

towards monitoring and safeguarding fish welfare at a company level (van de Vis et al., 2012). The system was named Fish Welfare Assurance System (FWAS) and, as Hazard Analysis and Critical Control Points, requires the identification of major hazards and of the Critical Control Points (CCPs) that need to be controlled in order to minimize or avoid the identified hazards. Hazards affecting fish welfare include water quality, feed amount and composition, husbandry practices and contaminants, among others. Suboptimal water quality, for example, is a stressor that can affect physiology, behaviour, growth rate, feed intake and conversion, and lead to pathological changes, damage to organs and mortality (MacIntyre et al., 2007). Environmental contaminants are one type of hazards affecting fish welfare, its health, chemical composition and quality with further implications for seafood safety and human health.

In accordance with van de Vis et al. (2012), this Thesis will follow the definitions of stress and stressors proposed by Wendelaar Bonga (1997), i.e. stress as a condition in which the dynamic equilibrium of an animal is threatened or disturbed as a result of the action of intrinsic and/or extrinsic stimuli, and stressors as the extrinsic stimuli.

Monitoring of Critical Control Points at a farm level is obviously essential and it requires the establishment of Operational Welfare Indicators that may be biotic, abiotic, managerial and environmental indicators. Each Operational Welfare Indicators (OWI) would have critical limits/target values. As in Hazard Analysis and Critical Control Points, critical limits are the maximum and the minimum values within which the corresponding indicator must be, at its corresponding critical control point, in order to prevent, eliminate or reduce a hazard to an acceptable level for the welfare of the fish (van de Vis et al., 2012). Examples of Operational Welfare Indicators (depending on the hazard of course) during the on-growing phase are: "Visual inspect for gill and skin lesions", "Observe feed intake and behaviour" and "Inspect fish for lesions and deviations from behaviour".

The Fish Welfare Assurance System and the Operational Welfare Indicators, proposed by van de Vis et al. (2012) provide a methodology that Webster (2001) claimed in his review was needed; namely, a method based on welfare-based quality assurance schemes with quality control ensured by independent audits. Audit protocols are based largely on the identification of the elements of good husbandry; therefore, what Webster (2001) proposed was ultimately needed was an independent audit to ensure that the outcome of the perceived elements of good husbandry are, in fact, good animal welfare.

It is the author's hope that this PhD Thesis contribute to monitoring welfare by providing a tool to non-invasively monitor fish behaviour and its deviations, thus aiding in implementing the Fish Welfare Assurance System systems and on-line welfare objective monitoring.

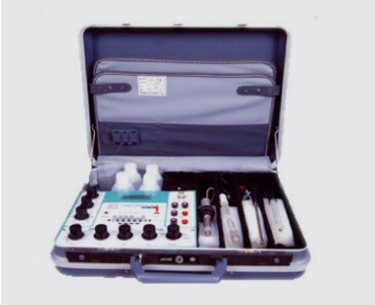
The contaminants that affect fish welfare and seafood safety are many and not all of them are known. The rest of the introduction will focus on some chemical contaminants which have been selected for the challenges their detection presents (see below), and among them methylmercury. Methylmercury has been targeted for the apparent increase in the number of seafood species in which it is being detected and the concern it awakens among consumers and food safety authorities.

1.3. Challenges in the monitoring of chemical contaminants

Monitoring and detection of chemical contaminants in the seafood chain remains most challenging for the following reasons (Eguiraun et al., 2015a):

- The cost of their analyses is usually high and only highly specialized laboratories perform them.
- Many of the contaminants consist not of one, but of "families" of compounds (i.e., a compound, its metabolites and/or its congeners) with different degrees of toxicity.
- The nature of chemical contaminants is very diverse: they may be organic or inorganic, natural or synthetic, the result of industrial activities (cosmetic and oil industries, mining activities), agricultural activities (pesticides, fertilizers), the aquaculture industry itself (veterinary treatments, rests of non eaten food pellets in decomposition) and of the habits of the human populations close to the production or harvesting area (use of medicines, hormones, recreational drugs).
- Only expected contaminants are tested (maliciously introduced, unexpected, novel and emergent contaminants commonly remain undetected until the wildlife, the farmed species or the consumers are severely affected).
- Novel contaminants are being detected in environmental, sewage and drinking waters at an increasing rate (Roose et al., 2011).

Different approaches can be use to monitor the presence of contaminants. One is the detection of a targeted contaminant (analytical methods), another is the detection of the effect of a targeted contaminant (bioassays and biosensors) and the third is the detection of the toxic effects of a sample (air, water, feed) in which one or many known and unknown contaminants may be present in unknown amounts (Figure 1.3).

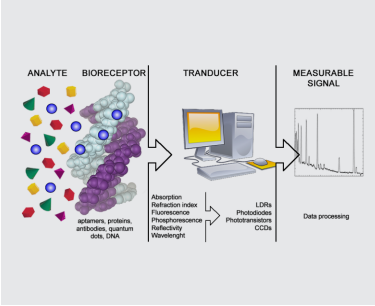


ID SUBSTANCES

- Specialized high technology labs
- Kits and portable kits

BIOASSAY – BIOLOGICAL ASSAY

- Determines the strength or biological activity of a substance (drug, pollutant) by comparing its effects with those of a standard preparation on a test system (organism, tissue, cell)



BIOSENSOR – BIOLOGICAL SENSOR

- Device made up of a transducer and a biological element (enzyme, antibody, bacteria...) where the bioelement interacts with the analyte and the biological response is converted into a electrical signal by the transducer

BIOLOGICAL WARNING SYSTEMS

- Any system of biological or technical nature deployed by an individual or group to inform of a future danger

Figure 1.3. Classical and modern approaches to detect contaminants, from top to bottom: identify the contaminants using analytical technique; bioassays and biosensors designed to identify targeted contaminants based on specific biological effects and Biological Warning Systems that will detect the integrated effect of any and all the contaminants affecting the biological system. Images from: https://en.wikipedia.org

In real life situations, contaminations are seldom due to one single compound and it is becoming increasingly evident that the number of novel substances with a potentially negative effect that must be monitored is increasing. Therefore, there is a need to develop new approaches to ensure our safety. To illustrate this, some cases of malicious and of emergent contaminants will be mentioned.

1.4. Malicious contaminants

Perhaps the most famous case of an unexpected maliciously introduced chemical contaminant was the use of melamine in feeds and foods (Sharma and Paradakar, 2010). The products had been manufactured in China, killed thousands of pets and the case was best documented in the USA (Dobson et al., 2008). It caused the deaths of at least six babies and the illness of about 300,000 more who consumed adulterated infant formula milk in China. The reason why melamine was added to foods and feeds was to increase the profit by falsifying the protein content of products. The amount of protein in foods and feeds is indirectly estimated by multiplying by a factor of 6.25 (for meat and feeds) the total nitrogen of the sample, which in turn is estimated by the Kjeldahl method (Tacon, 1987). When a large amount of the nitrogen in the sample originates from nitrogen-rich contaminants such as urea, melamine and their derivatives, the protein amount is wrongly overestimated. Fraudulent actors can then sell their products with much lower protein content and increase their profits. It seems that after the addition of urea was made illegal the addition of melamine became more popular. It was a practice that had apparently been taking place for years, but it was not suspected until deaths started to take place. The main reason for the deaths was that ingestion of melamine and its derivatives cyanuric acid and melamine cyanurate form insoluble crystals that precipitate in kidney tubules physically blocking them and inducing renal failure and death (Brown et al., 2007).

The scandal had global proportions: it originated in China but the deaths of pets were first detected in the USA and then contaminated products were found all over the world (including Australia, Canada, India, Hong Kong, Malaysia, New Zealand, Japan, Switzerland, Taiwan, The Netherlands and USA) and in products from multinational reputed brands such as Cadbury, Heinz, Nestlé, Lipton and Tesco. Food products such as chocolate, cookies, coffee, eggs, pork, marketed fish fillet, chicken and pet food, swine, poultry and fish feeds were contaminated, suggesting that adulteration of feeds had been a common practice for some years prior to its detection (Brown et al., 2007; Dobson et al., 2008; Sharma and Paradakar, 2010). As a consequence of this scandal, several analytical methods have been developed and become obligatory to ensure that foods and feeds are free of this contaminant (Liu et al., 2012).

In cases such as this, where the contaminant is unexpected, introduced purposely and fraudulently and completely unrelated to any of the materials expected to come in contact with the product, its identification is extremely unlikely: in one of the earliest studies trying to identify pet deaths (Brown et al., 2007) most of the initially collected necropsy samples were not tested for melamine but, interestingly, the few that were tested turned out to be positive.

1.5. Emergent chemical contaminants

Many of the organic pollutants currently being monitored in European waters are endocrine disruptors (Bevan et al., 2012; Roose et al., 2011), i.e., chemicals that exhibit hormone-like effects or that interfere with the hormonal signals of an organism. The structure of these compounds mimics that of oestrogens and when ingested by male organisms, these may develop female characters, a fact that severely hampers their reproductive capability and may end up in population collapse (Kidd et al., 2007). A possible relationship with development of diabetes type II has also been postulated (Magliano et al., 2014).

In addition to these, novel emergent chemical contaminants are being identified with increasing frequency in the environment, including recreational and prescription drugs, beauty care products and the metabolites of all these compounds. Monitoring of this kind of contaminants is a priority target of the European Water Framework Directive and of the EU Marine Strategy Framework Directive [see (Allan et al., 2006)] but in most countries analyses for their detection and monitoring are still not implemented and they are not included in programmes for feed and food safety.

The presence in raw sewage water of recreational drugs and their metabolites has reached a point where sewage epidemiology may become an effective monitoring tool to estimate the consumption of classical (Irvine et al., 2011; Lai et al., 2011; Pinkiewicz, 2012; van Nuijs et al., 2011) and novel (Reid et al., 2014) illicit drugs. It has been detected an annual increase in the number of new psychoactive substances formally notified for the first time in Europe through the EU Early Warning System from 13 substances in 2008 up to 73 in 2012 (EUROPOL, 2012). The efficient removal of these substances greatly depends on the methods used to treat the wastewater prior to its discard or recirculation: conventional activated sludge efficiently removed (from about 80% to over 90%) cocaine and its metabolites, opiates and related compounds and ketamine, but its efficiency varied for amphetamine-like substances (>90% some, 50% others), cannabinoids (90% some, 40% others) and practically did not remove nor-LSD (van Nuijs and Covaci, 2012).

Also the presence of prescription drugs in wastewater is becoming so common that some of them can be used to estimate the size of a given population; for example Lai et al. (2011) concluded that the amount of atenolol (a selective 1 receptor antagonist used primarily for the treatment of cardiovascular diseases) in sewage water in Queensland, Australia seemed to be appropriate for that particular location because it was used on a daily bases by 1 - 3% of the population in Australia; it is not effectively removed in wastewater treatment plants (which makes it persistent and mobile in the sewers); and finally because the age group of atenolol consumers matched the age group of the population to be estimated. The same study also detected carbamazepine, gabapentin, hydrochlorothiazide and venlafaxine in the wastewater of Queensland, although they were not suitable to estimate the size of the population. Carbamazepine (an anticonvulsant and mood-stabilizer used primarily in the treatment of epilepsy, bipolar disorder and trigeminal neuralgia, attention-deficit hyperactivity disorder, schizophrenia, phantom limb syndrome, complex

regional pain syndrome, borderline personality disorder, and post-traumatic stress disorder) is a stabilizer of the inactivated state of voltage-gated sodium channels. Gabapentin is a GABA analog developed to treat epilepsy, and currently used also to relieve neuropathic pain; hydrochlorothiazide is frequently used for the treatment of hypertension, congestive heart failure, symptomatic oedema, diabetes, renal tubular acidosis, and the prevention of kidney stones and reduces blood volume by acting on the kidneys to reduce sodium reabsorption in the distal convoluted tubule; and venlafaxine is an antidepressant of the serotonin-norepinephrine reuptake inhibitor class, used primarily for the treatment of depression, general anxiety disorder, social phobia, panic disorder and vasomotor symptoms.

In a USA pilot survey of the occurrence of pharmaceutical and personal care products in fish, Ramirez et al. (2009) reported to have identified the antidepressant sertraline at levels of 19 ng/g in fillet and 545 ng/g in liver of fish as well as the synthetic musks commonly used in cosmetic products galaxolide (2,100 ng/g fish) and tonalide (290 ng/g fish). Other substances whose effects on wildlife are still undocumented include the artificial sweetener acesulfame, which is excreted with practically no alteration and persistent in wastewater (Lai et al., 2011) and caffeine, detected in sewage water and reported to be a useful tracer for faecal contamination (Buerge et al., 2003; Potera, 2012).

Recently, Robles-Molina, et al. (2014) published a liquid chromatography high-resolution mass spectrometry method for the simultaneous determination of over 400 multi-class priority and emerging pollutants detected in environmental waters which included 105 multiclass pharmaceuticals (analgesics/anti-inflammatories, antibiotics, lipid regulators, β-blockers, antiepileptic/psychiatrics, ulcer healings, diuretics, hormones and bronchodilatadors), life-style products (caffeine, nicotine), 21 drugs of abuse and their metabolites, 279 pesticides and some of their more relevant metabolites, nitrosamines, flame retardants, plasticizers and perfluorinated compounds.

Therefore, the candidate considers, that this new situation with a large amount of unexpected contaminants in the environmental waters, demands a new approach for their detection and monitoring of their effects on fish welfare, behaviour and seafood safety, i.e. a paradigm shift in seafood safety assurance. Figure 1.4 shows a classification of pollutants attending their origin.

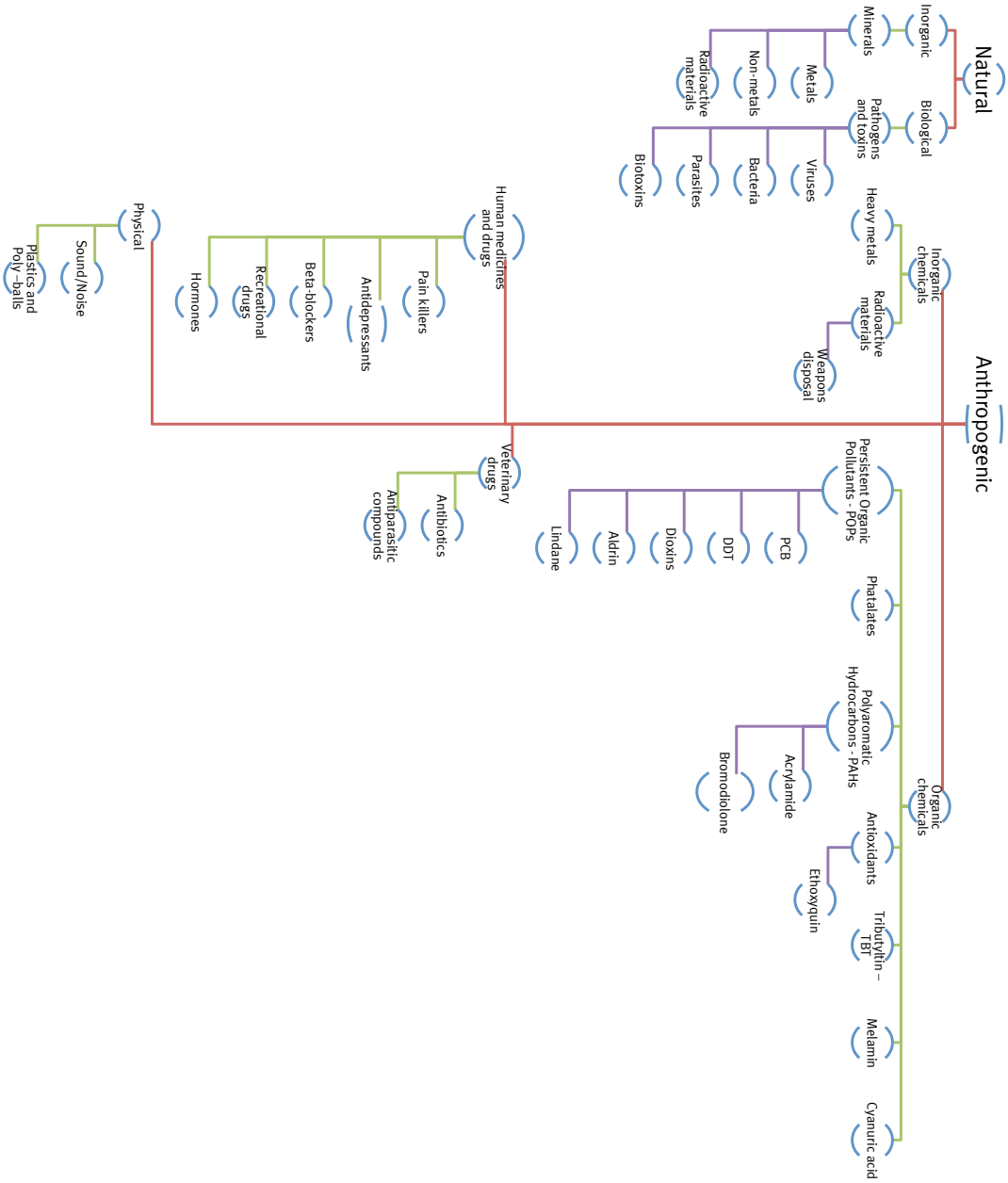


Figure 1.4. A schematic representation of pollutants attending their origin.

1.6. The need for a paradigm shift in seafood safety assurance

The current approach to ensure seafood safety, following the dictates of a strict Hazard Analysis and Critical Control Point system, includes a thorough description of the product and ingredients; the identification of all possible and relevant hazards and their routes of entrance; the establishment of critical points and critical limits for each hazard; monitoring and validation methods; preventive measures to avoid the entrance of contaminants in the production chain and corrective actions for those cases when deviations from the determined parameters take place. Although Hazard Analysis and Critical Control Point procedures should include all types of hazards, i.e., chemical, physical and biological or microbiological, the effectiveness of these procedures to control chemical hazards has been severely limited (as already mentioned above) by the variety of chemicals and their sources and the high cost of chemical monitoring (Ropkins and Beck, 2003). It is certainly not possible to define any of these parameters for unknown and/or unexpected substances.

In this context, we propose to introduce a shift in the manner seafood safety is envisioned (Eguiraun et al., 2015a). A paradigm also applicable to the production of any other food and feedstuff and to animal welfare monitoring that is based on the following facts:

- It is not possible nor desirable, to analyse every single potentially contaminating compound (for example, some compounds, or their levels, may not represent a risk in seafood production).
- It is not possible to monitor the presence or amount of unknown substances even if they are extremely harmful to the fish or to man.
- It is possible to detect a substance's effect on a biological system, such as fish, by examining the distress and/or behavioural changes induced on it and using those signals as a warning signal indicating that something is altered or wrong, including the welfare of the animal [see reviews (Allan et al., 2006; Bae and Park, 2014; Environmental Protection Agency, 2005)].

In fact, the fish will integrate all the effects of the substances and conditions to which it is exposed: if the substances do not have a relevant effect, nothing will be noted; but if on the other hand there is something, known or unknown that influences the fish, its organism will register it, and, hopefully, it should be possible to develop a tool to detect those changes in the fish behaviour that indicate the exposition to a stressor and potentially harmful agent.

Although this approach is somehow similar to the use of biosensors, there is a crucial difference: biosensors are chosen for their suitability to respond to certain contaminants, but they do not respond to all relevant contaminants; on the other hand, the whole organism produces responses that are not necessarily contaminant-specific but reflect the real effect of the total mixture of substances on the animal (Roose et al., 2011). Also, biosensors are not intended to detect the effects of interacting substances or of substances with a positive effect.

1.6.1. Introduction to Biological Warning Systems (BWSs)

Implementation of the use of the entire animal as a monitoring device presents a clear advantage, and indeed it constitutes the principle behind the use of Biological Warning Systems (BWSs).

A BWS is any biological system set up to inform about the presence of a danger so that whoever set it up can prepare for the danger and react accordingly to avoid, reduce and/or control it. The use of higher organisms, fish in particular, for on-line toxicity detection of water quality in a Biological Warning System has been the subject of some reviews (Bae and Park, 2014; van der Schalie et al., 2001). Later on, in Chapter 3, a state of the art in the topic of Biological Warning Systems is presented.

Extending the proposal of Allan et al. (2006) to include BWSs among the methodologies considered suitable to implement the EU's Water Framework Directive, we suggest that these methodologies may, on their own right, find an application in aquaculture safety monitoring programs and have the potential to become a core method in animal welfare and product safety and quality monitoring. BWSs will detect distress signals sent by the animal, distress that may be caused by contaminants, diseases and parasites, but also by stressors more difficult to identify and quantify such as suboptimal conditions including fish density, light intensity and wavelength, photoperiod, background colour, tank design or feed formulations, the lack of key nutrients, temperature variations and others.

The complex interplay of different factors/chemical substances and the need to consider the fish themselves as the main system is illustrated by the case of methylmercury (MeHg) and selenium (Se). It has become apparent that there is no straightforward relationship between the amount of methylmercury in seafood ingested and methylmercury toxicity in humans (Ralston et al., 2008; Raymond et al., 2012; M. Yamashita et al., 2013). This has recently been explained because methylmercury, due of its high affinity for selenium, exerts its toxicity, by inactivating selenoproteins, which have a strong antioxidant activity (Ralston et al., 2008; Raymond et al., 2012; M. Yamashita et al., 2013; Y. Yamashita et al., 2013). Interestingly, marine fish and particularly tuna are very rich in the recently discovered selenium-containing molecule called selenoneine (Yamashita and Yamashita, 2010; Y. Yamashita et al., 2013) that has been shown to have a methylmercury detoxifying effect and it does not seem to be toxic in concentrations that would have caused selenosis if the source of selenium was a number of other selenium compounds (M. Yamashita et al., 2013). Thus, neither the level of methylmercury nor that of selenium individually would be indicators of the potential toxicity of a product, since the real effect would depend both on: the selenium-methylmercury ratio and on the chemical nature of the selenium compound.

On the other hand, it is possible that fish may display signs of stress making the Biological Warning System go off, but analyses of known potential stressors would not identify the cause. This would illustrate the case of a suspected novel and/or unexpected stressor or contaminant in the environment. At this point one would need to initiate a detective work until the cause is identified, analysed and its risk evaluated.

The affected fish should be isolated as potentially tainted and in this manner avoid the entrance of novel or malicious contaminants into the food chain.

Generally, a Biological Warning System will be better suited to predict and give a first alarm signal of the toxic substances entering the seafood production chain in fish farming than chemical analyses because chemical analyses of the levels of, for example, each metal (using again the case of MeHg and Se) would not provide a suitable answer, lacking the integrated response that is characteristic for a system, while unknown substances will not be tested for. In the same way, biosensors will also present the need to develop a specific biological element for each contaminant and organism. Moreover, and given that the contaminant may originate in the water and/or in the feed, each test will require different extraction and testing procedures, and probably will never be robust enough to detect alterations that are not contemplated in the design phase.

Once designed, tested and validated; a Biological Warning System should be implemented within the Hazard Analysis and Critical Control Points and Fish Welfare Assurance Systems, as the monitoring procedure it is, and a series of corrective actions should be listed in case an alarm is initiated. To achieve this purpose, a good Biological Warning System must fulfil a series of requirements listed by Hasan et al. (2004) and summarized as:

1. Provide a fast response.
2. Screen for a number of contaminants.
3. Be automated.
4. Permit remote monitoring.

In addition, if such a system is to be implemented within a production system, it would need to fulfil the following requirements:

- Provide the warning early enough to allow to take remedial actions.
- Be cost effective and efficient.
- Not require highly specialized personnel or equipment (i.e. suitable to be used by personnel only after a short training).
- Be robust, i.e., capable of operating under a wide range of working conditions.
- Give minimal false positive and false negative results.

1.7. Understanding, defining and dealing with a system

In his 1990 work, Broom already used the concept of system when he related the concept welfare to the idea of “systems for coping with difficulties during life”. The terms “system” and “biological system” have already been used in this introduction, but they have not yet been defined.

A system will be considered as a black box with inputs and outputs. The outputs will be defined in relation to the inputs and the intrinsic dynamic of what the black box contains. As big problems require big boxes, it is possible to divide those big boxes in smaller ones. If they are properly defined and interconnected, it can be assumed that the sum of the small boxes should be comparable to the big one, i.e., a big problem has been divided into smaller problems, which are intended to be easier to solve. This apparently straightforward methodology is the core of Systems Engineering (see Bhatikar et al., 2000; Cellier, 2013; Kuehn and Gross, 2013; Ljung, 2001; Ma and Zuo, 2014; Matko et al., 1992; Ogata, 2009; Recktenwald, 2000; Schuster, 2010; Woods and Lawrence, 1997; Zhang et al., 2007 for further details).

The degree of abstraction determines the complexity and the accuracy of the approach, but, there must always be a compromise between the degree of abstraction and the feasibility (cost and ease of execution) of its design and implementation. There are three key steps in the initial part of this process: data collection, data processing and knowledge inference.

Not surprisingly, the quality of the data is the most critical part of the entire process. Depending on the complexity of the system, one may encounter the challenges characteristic for "big data", namely data capture, curation, storage, search, sharing, transfer, analysis and visualization.

Knowledge can be inferred from the data by using either Classical or Intelligent Techniques. Classical Techniques make use of traditional control systems methodologies that merge classical modelling techniques in continuous or discrete time scales and events and/or stochastic modelling techniques. There are two main types of classical techniques: those based on Theoretical Models and those based on Experimental Models. Theoretical modelling techniques are fuelled with strong mathematical background including differential, linear and non-linear equations. These classical methods are the ones that have traditionally been applied and are suited to understand and resolve most of the real world problems.

When the relevant data required to build up models are complex and come from different disciplines they need to be simplified by using data pre-treatment and processing methods. For example, principal component analysis (PCA), that serves to identify which variables are relevant and which are not, which ones constitute noise, and/or provide redundant information. The next step is the storage into databases of all the relevant, pre-processed data that are going to be used to infer knowledge about the system.

Finally, there is a need to obtain relevant information from the database, i.e. the knowledge inference procedure that leads to a mathematical model suitable for prediction and/or control of the system. It must be noted that very complex processes are sometimes not successfully controlled or predicted in all of their working range and the designer must identify the so called Operational Working Point/Points of the system (i.e. the operational conditions under which the system is stable) and deal with it/them.

The final step is to develop a control procedure that, using the information received from the monitoring process, automatically regulates the necessary conditions to preserve the normal or desired status of the system and deals with the deviations, if registered, either as programmed, or following an intelligent approach.

Additionally, when dealing with complex systems and problems, the remaining unexplained variability must also be accounted for, explained and modelled. For that, one must use the so-called Intelligent Techniques. Unlike classical methods, intelligent techniques are suited to work with the uncertainty, constrictions and restrictions characteristic of nature itself. Intelligent techniques can in turn be divided into two types: those based on Biological Models (BM) and the ones based on Knowledge Models. The most successful and popular intelligent systems based on Biological Models are the Artificial Neural Networks (ANN) and all their variants. Basically, Artificial Neural Networks are algorithms able to learn, generalize and abstract. Biological Models also includes Evolutive Techniques, such as Genetic Algorithms, Evolutive Strategies and Genetic Programming. Roughly, these techniques are based on a natural selection process that mimics biological evolution. The algorithm repeatedly modifies a population of individual solutions. At each step, the genetic algorithm randomly selects individuals from the current population and uses them as parents to produce the children for the next generation. Over successive generations, the population "evolves" toward an optimal solution or an accepted suboptimal solution. Knowledge Models (KM) comprise Expert Systems (computer systems that emulate the decision-making ability of a human expert) and Rule Based Systems (systems that store and manipulate knowledge to interpret information in a useful way from a starting set of data and rules).

Finally, it must be mentioned that building an online monitoring system will provide the supervisor with the ability to filter a large amount of information and return the necessary information-knowledge to take optimal decisions. It must also be noted that an online monitoring system does not necessarily have to be also a real-time monitoring system. Strictly speaking, the real-time concept involves that any activity of data processing and return of information must respond to the externally generated stimulus in a specified, finite and short time interval (Laplante et al., 2011). Thus, real-time systems have to be built with special software requirements, which make them more expensive and difficult to implement (Burns and Wellings, 2009). A brief summary of this methodology is depicted in Figure 1.5.

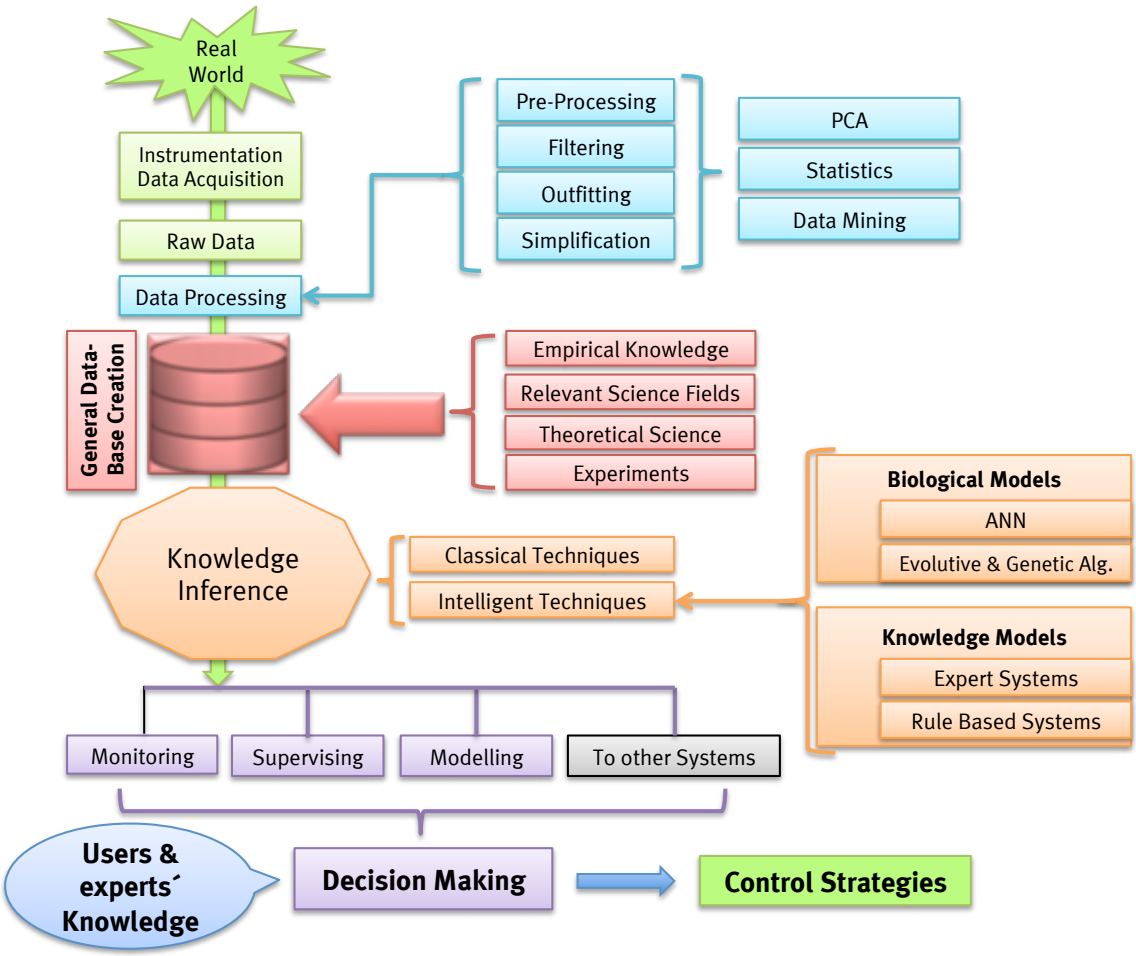


Figure 1.5. Topology of a proposed Biological Warning System. Theoretical representation of how a Biological Warning System for aquaculture industry might be.

1.8. Biological systems and fish farming

Biological systems are extraordinarily complex, they are regulated by interacting mechanisms that operate across multiple spatial and temporal scales and their understanding requires the integration of computational and experimental research (Kitano, 2002). Therefore, in the candidate's opinion, it is essential to apply a systems approach to optimize aquaculture production, fish welfare and product quality, given that each farm is indeed a biological system. The output variables of biological systems often have complex fluctuations that may contain information about their intrinsic dynamics, and the time series they generate often contain deterministic and stochastic components that are not properly addressed by traditional approaches (Costa et al., 2005, 2002). In order to face these challenges creative solutions are needed.

For fish farming, basically it should be a system which automatically regulates the necessary conditions to preserve the normal or desired status of the production system and deals with the deviations, if registered, either as programmed, or following an intelligent approach. In that regard, the supervising system should be able to respond fairly rapidly if serious deviations start occurring suddenly: for example as result of an accident (a spillage) or a contaminated batch of feed. The speed of the response may be critical to save the affected units and preserve their welfare and quality. To implement such an approach, relevant scientific data from a variety of research fields and control analyses need to be gathered and complemented with empirical data about farming of the targeted species. It is important to keep in mind that the empirical knowledge of the farmer must never be underestimated.

In addition, fish farming will probably not demand the implementation of real-time architecture and, in fact, an on-line monitoring system with a web-based service will allow the farmer to manage all the necessary information wherever he or she might be.

1.9. Hypothesis

The present Thesis is based on the following Hypothesis:

A fish system will behave as a biological sensor integrating biological and physiological responses to external stimuli and the response to those stimuli can be quantified in a non-invasive manner. Furthermore, the quantified alteration in the fish system has the potential to serve as a tool for on-line fish welfare monitoring.

1.10. Objectives and contributions

The main objective of this Thesis was to design and develop a tool that using a fish system as a biological sensor, had the potential of being implemented within a Biological Warning System in the aquaculture industry.

The fish species selected for the empiric work was European seabass (*Dicentrarchus labrax*) due to its relevance for Southern European aquaculture and the need to non-invasively monitor and control relevant variables during its production, in particular those relating to seafood safety and to the welfare of the fish.

Secondary objectives dealt with the analysis and evaluation of the proposed tool. Specifically, it was selected to quantify how the fish system reacted under the following conditions:

- A variable number of fish in the system.
- The addition of selenium to the fish system.
- The exposition to a neurotoxic contaminant named methylmercury to the fish system.

The third and last objective of this Thesis was to build up a first-version of a model that might be -in future works, and after further development, testing and validating- input into a Knowledge Database that would in turn serve as the information center for a Biological Warning System.

With regards to the first and secondary objectives, the main contributions of this Thesis are:

- A new working methodology based on the systems engineering philosophy has been developed.
- A inexpensive, adaptable and non-invasive tool-framework based on image analysis to monitor fish responses suitable to be implemented in an on-line early Biological Warning System has been developed.
- A sensor unit has been developed consisting of using of the fish group movement pattern as a response to a perturbation. Deviations in this movement pattern from a desired working point should alert about undesirable perturbations.
- Development of a series of non-linear signal analysis techniques suitable to analyse the trajectory signal of the fish group.
- Development of a mathematical model of the fish system's response to an external perturbation.
- Testing of the developed methodology and the tool on experimental cases.

1.11. Ethical statements

All experimental protocols and procedures conducted with animals in the present Thesis were carried out under the approbation of The Ethical Committee of the University of the Basque Country UPV/EHU for Animal Welfare No. CEBA/285/2013MG.

1.12. Scientific output

This Thesis covers the result of three years of work and the work performed has been published, presented and/or submitted to the following scientific journals and conferences.

1.12.1. JCR peer reviewed scientific journals

- Eguiraun, H., Izagirre U., Lopez de Ipiña, K., & Martinez, I. (2015). Evolution of the Shannon entropy in a fish system (European seabass, *Dicentrarchus labrax*) in response to its density. Submitted.
- Eguiraun, H., Izagirre, U. & Martinez, I. (2015). A paradigm shift in safe seafood production: from contaminant detection to fish monitoring - Application of Biological Warning Systems to Aquaculture. Trends in Food Science and Technology, 45, pp 104-115. Elsevier. DOI: 10.1016/j.tifs.2015.01.007.
- Eguiraun, H., Lopez-de-Ipina, K., & Martinez, I. (2014). Application of entropy and fractal dimension analyses to the pattern recognition of contaminated fish responses in aquaculture. Entropy, 16, pp 6133-6151. DOI: 10.3390/e16116133.
- Kalogerakis, N., Arff, J., Banat, I.M., Broch, O.J., Daffonchio, D., Edvardsen, T., Eguiraun, H., Giuliano, L., Handå, A., López-de-Ipiña, K., Marigomez, I., Martinez, I., Øie, G., Rojo, F., Skjermo, J., Zanaroli, G., Fava, F. (2014). The role of environmental biotechnology in exploring, exploiting, monitoring, preserving, protecting and decontaminating the marine environment. New Biotechnology, 32, 1. pp 157-167. DOI: 10.1016/j.nbt.2014.03.007. ISSN: 1871-6784

1.12.2. Refereed published conference proceeding

- Eguiraun, H., Martinez, I. (2015). Evolution of Shannon entropy in a fish system (European seabass, *Dicentrarchus labrax*) during exposure to sodium selenite (Na_2SeO_3). In Proceedings of the 2nd International Electronic Conference on Entropy and its Applications (ECEA), pp 1-7. 15th-30th November 2015; Sciforum Electronic Conference Series, Vol. 2, session Complex Systems (C006). DOI:10.3390/ecea-2-C006
- Eguiraun, H., Lopez de Ipiña, K., & Martinez, I. (2015). Evolution of Shannon entropy in a fish system (European seabass, *Dicentrarchus labrax*) during methylmercury post-exposure. IEEE 4rd International Conference and Workshop on Bioinspired Intelligence (IWOBI), pp 59-63. 10th - 12th June 2015. Donostia, Spain. DOI: 10.1109/IWOBI.2015.7160145.

- Eguiraun, H., Lopez de Ipiña, K. & Martinez, I. (2014). Non-invasive methods based on non-linear analyses to monitor fish behaviour and welfare. 48th Congress of the International Society for Applied Ethology (ISAE), p 114. July 29th - August 2nd 2014, Vitoria-Gazteiz, Spain. DOI: 10.3920/978-90-86-86-797-4.

- Eguiraun, H., Lopez-de-Ipina, K. & Martinez, I. (2014). Discrimination of contaminated fish responses by fractal dimension and entropy algorithms. In: IEEE Conference Publications-Proceedings of the 2014 International Work Conference on Bio-inspired Intelligence (IWOBI), pp 173-177. 16th - 18th July 2014. Liberia, Costa Rica. DOI: 10.1109/IWOBI.2014.6913959.

1.12.3. International conferences

The main author and the presenting author appear in bold and underlined respectively.

- **Eguiraun, H.**, Martinez, I. (2015). Evolution of Shannon entropy in a fish system (European seabass, *Dicentrarchus labrax*) during exposure to sodium selenite (Na_2SeO_3). 2nd International Electronic Conference on Entropy and its Applications (ECEA), Sciforum Electronic Conference Series, Vol. 2, session Complex Systems (C006). 15th - 30th November 2015. Online presentation.

- **Eguiraun, H.**, Martinez, I. (2015). Evolution of the Shannon entropy in a fish system (European seabass, *Dicentrarchus labrax*) in response to its density. European Aquaculture Society, (EAS). 20th - 23th October 2015. Rotterdam, The Netherlands. By Invitation to Special Session PS21 Fish Welfare. Oral presentation.

- **Martinez, I.**, Eguiraun, H. (2015). Use of fish as a Biological Warning System (BWS) in aquaculture to monitor fish health and welfare, product quality and safety. European Aquaculture Society, (EAS). 20th - 23th October 2015. Rotterdam, The Netherlands. By Invitation to Special Session PS21 Fish Welfare. Oral presentation.

- **Eguiraun, H.**, Lopez-de-Ipina, K. and Martinez, I. (2015). Evolution of Shannon entropy in a fish system (European seabass, *Dicentrarchus labrax*) during the recuperation period after exposure to methylmercury. IEEE 4rd International Conference and Workshop on Bioinspired Intelligence, (IWOBI). 10th - 12th June 2015. Donostia, Spain. Oral presentation.

- **Eguiraun, H.**, Izagirre, U., Vitalle, J., Marigomez, I., Soto, M., Lekube, X., Erdaide, O. and Martinez, I. (2014). Fish behaviour analysis for Environmental Monitoring. 3rd Annual World Congress of Aquaculture and Fisheries, (WCAF). 16th - 18th October 2014. Dalian, China. Poster presentation.

- **Eguiraun, H.**, Lopez-de-Ipina, K. and Martinez, I. (2014). Non-linear fish behaviour analysis in response to contaminants: Entropy and Fractal approaches. 3rd Annual World Congress of Aquaculture and Fisheries, (WCAF). 16th - 18th October 2014. Dalian, China. Oral presentation.

- **Martinez, I.**, Eguiraun, H., Lekube, X., Soto, M. & Marigomez, I. (2014). Trends in European aquaculture research to ensure high quality and safe seafood products. 3rd Annual World Congress of Aquaculture and Fisheries , (WCAF). 16th - 18th October 2014. Dalian, China. Oral presentation.

- **Eguiraun, H.**, Lopez-de-Ipina, K. & Martinez, I. (2014). Discrimination of contaminated fish responses by fractal dimension and entropy algorithms. IEEE 3rd International Conference and Workshop on Bioinspired Intelligence, (IWOBI). 16th - 18th July 2014. Liberia, Costa Rica. Oral presentation.

- Eguiraun, H., Bwye, G., Erdaide O., Izaguirre U., Lekube X., Lopez de Ipiña, K., **Martinez, I.** (2013). Response to methylmercury of European seabass (*Dicentrarchus labrax*) pre-exposed to sodium selenite -Non-destructive methods to recognize contaminated fish and fish welfare. 10th International Symposium on Selenium in Biology and Medicine.14th - 18th September 2013. Berlin, Germany. Poster presentation.

- **Eguiraun, H.**, Lopez de Ipiña, K. & Martinez, I. (2013). Designing a framework for the application of systems biology to the fish farming industry. 14th International Conference on Systems Biology. 29th August to 4th September 2013. Copenhagen, Denmark. Poster presentation.

- **Eguiraun, H.**, Bwye, G., Lopez de Ipiña, K. & Martinez, I. (2013). A systems approach to predicting quality and safety in the aquaculture industry. European Aquaculture Society, (EAS). 9th - 12th August 2013. Trondheim, Norway. Oral presentation.

- **Bwye, G.**, Eguiraun, H., Lekube, X., Izagirre, U., & Martinez, I. (2013). Effect of the addition of sodium selenite and/or methyl mercury on the protein make up of European seabass (*Dicentrarchus labrax*) white skeletal muscle. European Aquaculture Society, (EAS). 9th - 12th August 2013. Trondheim, Norway. Oral presentation.

1.12.4. Grants

The author was the holder of a European Economic Area (EEA) Researcher Mobility and Co-operation Grant NILS Science and Sustainability Programme (ES07), ABEL-IM-2014B Call, in order to visit AMOS - The Center for Autonomous Marine Operations and Systems of the Norwegian University of Science and Technology (NTNU), Trondheim, Norway.

The subject of the grant was: Systems Aquaculture-Implementation of Biological Warning Systems within intelligent aquaculture structures (SYSAQUA).

CHAPTER 2
RESEARCH METHODOLOGY

2.1. Introduction

The objectives of this Thesis are designed to serve a wide range of scientific fields and methodologies such as biology, ethology, environmental monitoring, fish welfare, food safety, systems engineering and non-linear signal processing.

This chapter formulates the research questions that have motivated the Thesis and explains the research plan and its benefits. It also describes briefly the research methods used as well as the limitations of the present work.

2.2. Research questions

The long-term objective of the work addressed by this Thesis is the development of a Biological Warning System based on the response of a fish system. Thus, the main research questions of the present Thesis are:

- Does a fish system subjected to a perturbation/stressor alters its behaviour?
- Are those differences measurable? And are they measurable in a non-invasive manner?

If the answers to these three questions are positive, it follows that the fish themselves may be used as a Biological Warning System. Then, assuming that the differences are measurable in a non-invasive manner, it may happen that exposure of the fish system to some perturbations (for example contaminants) could cause the death of some of the fish. Therefore the system itself is suffering an alteration, i.e. the number of fish. Consequently, a third research question should be:

- Does the number of fish affect the response of the system?

Finally, all this knowledge should applied to construct a mathematical model suitable to use in an on-line fish monitoring system based on a Biological Warning System. Thus the fourth and last question was:

- Is it possible to build a mathematical model that would react in response to differences between the measured and the desired behaviours of the system?

2.3. Research plan

This Thesis follows a methodology in which the research is a sum of scientific and empirical approaches. The scientific approach is based on theory and observations and it covers the first part of the Thesis resulting in a tool that is tested in the second part of the Thesis. This second part comprises the empirical approach where the tool is applied to different case studies.

Consequently, the research is divided into four steps as formulated by Glass (1995): informational, propositional, analytical and evaluative. These steps are described next.

2.3.1. Informational phase

This phase has two aims. First, to collect information in order to have an outline of the current knowledge involved in the problem domain. Second, to formulate the research questions (listed in paragraph 2.2), and to identify the implications of this research for practice and/or policy. Thus, this phase comprises the search, review and analysis of the scientific literature and engineering solutions regarding the topics of the problem domain as well as the links among them: underwater video recording, fish tracking, image processing, fish behaviour, shoaling principles, interactions with pollutants, and existing Biological Warning Systems, among others (Chapters 1, 2 and 3).

2.3.2. Propositional phase

In this phase a solution to the main research question is presented, developed from the information gathered in the informational phase. The result of this phase is the Working Hypothesis (Chapter 1).

2.3.3. Analytical phase

The main purpose of this phase is to test the validity of the Working Hypothesis using scientific analytical methods, some of which have already been identified in the Informational Phase. In the present work, it consists of developing and testing a tool to monitor the response of a fish system under different experimental conditions: the case studies (Chapters 4, 5 and 6). The three case studies examined in this work are independent from each other. Thus, the nomenclature used refers to a “case” or “C” when a comparative study is made with inclusion of contaminants (Chapters 4 and 6). The word “tank” or “T” is used when the study uses replicate tanks as it happens in Chapter 5.

2.3.4. Evaluative phase

Based on the evidence of the knowledge acquired in the previous phases, a mathematical model is constructed. The building of the model was initially carried out using only data acquired in one of the case studies (variation in the number of fish) and it was tested/verified with data obtained from the second case study (exposure to chemical substances). The fitting of the date of the second (test) case study on the initial model constructed with only the data from the first case study was so good that all the data, from both case studies, were included in the construction of the final model. The evaluative phase is covered in Chapter 7 of the present Thesis.

2.4. Benefits of the research plan

The aforementioned research plan offers several benefits:

- From the scientific point of view, the research plan offers a starting point to understand the intrinsic dynamics of the system formed by the fish and everything else in their surrounding environment. Understanding all together, as a unique system, is necessary to understand the overall nature of the phenomena within.
- From the methodological point of view, this Thesis includes various relevant case studies. The case studies have provided real implementation and feedback about the proposed tool and the phenomena examined. This part has been essential to understand some of the limitations of the present study, which are explained in last part of the present Chapter.
- From the applied point of view, the potential implementation into real aquaculture setups of the tool developed in the present Thesis and the methodology used in the laboratory experiments was examined thanks to a Grant entitled "Systems Aquaculture: Implementation of biological systems within intelligent aquaculture structures (SYSAQUA)", funded by the European Economic Area (EEA) Researcher Mobility and Co-operation Grant, named NILS Science and Sustainability Programme, to the author of this Thesis which allowed him to stay at the Centre for Autonomous Marine Operations and Systems (AMOS) of the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway for three months.
- From the diffusion of research point of view, the research methodology of this Thesis has allowed the author to gradually improve the quality, quantity and range of the publications derived from it. The findings have been presented to various international peer-reviewed conferences and published and/or submitted to several JCR peer-review scientific journals (Chapter 1).
- From the social point of view, this work has allowed the author to greatly expand his network of scientific contacts. This fact will hopefully allow the author to collaborate and participate in different national and international research projects and events in the future.

2.5. Specific research methods used in this Thesis

The principles of case study methodology are followed in the experimental part of this Thesis. The case study method, widely used in the scientific world, was defined by Yin (1993) as "an empirical inquiry that investigates a contemporary phenomenon within its real-life context, when the boundaries between phenomenon and context are not clearly evident, and in which multiple sources of evidence are used".

The case study method is often used to generate an inductive thinking. Its results relate directly to the common everyday experience and facilitate the understanding of complex real-life situations. Its main advantage is the easiness to apply it to real life situations.

The present Thesis follows the steps proposed and established by Stake (1995) and Simons (1980) in order prepare and conduct a case study successfully. Those steps are:

- Formulate the research questions.
- Select the case study.
- Determine data collection and analysis techniques.
- Fieldwork for collecting data.
- Analyse and evaluate the data.
- Prepare the report.

Three case studies are analysed in the present Thesis in order to clarify the complexity of the biological system subject of the study. These kind of systems present an intrinsic and fluctuating dynamic that consists of a large amount of time series which contain both deterministic and stochastic components (Costa et al., 2005). In the present work, these time series are obtained by image processing and they consist of signal and noise. Classical approaches are not able to quantify the complexity of these systems; that is why real world applications and noisy environments often require alternative techniques leading to improved systems. The combination of nonlinear and linear modelling and/or features has led to higher and more robust performance, something particularly promising for solving complex tasks in real environments (Travieso and Alonso, 2013). Thus, in order to capture all the richness of complex biological systems into theoretical models, applied mathematics and computing must be used (Kitano, 2002; Spasic et al., 2012). It follows a brief description of the most relevant ones used in this Thesis.

2.5.1. Image processing

Video sequences are transformed into image sequences for feature extraction. The video is recorded in RGB (Red Green Blue) colour model and it is converted into 24 frames per second (fps) image sequences, which are cleaned and binarized for

feature extraction. Briefly explained, optical flux is generated by frame differentiation and the RGB images are converted into grey scale. Once the images are in black and white (B/W) they are cleaned through morphologic operations (closing, aperture and cleaning operations) for object detection. Object detection and consequent feature extraction was performed by detecting connected components in eight contiguous neighbourhoods. For further details see Chapter 4 and consult references Davies (2012), Hornberg (2007) and Myler (1999).

2.5.2. Cluster estimation

Once the objects within each image are located, their centres are determined. Then, the centroid formed by all of those centres is computed for every image and along the entire image sequence by using k-means clustering algorithm based on Euclidean distance.

The k-means clustering algorithm is a partitioning method that operates on actual observations and creates a single level of clusters, treating each observation in the data set as an object having a location in space. It finds a partition in which objects within each cluster are as close to each other, and as far from objects in other clusters, as possible. Depending on the kind of data to cluster, it is possible to use different methods to calculate the distances. The best results for the current problem were obtained using Euclidean distance, which is widely used, as in the works by Spath (1985) and Goncalves et al. (2007).

In each image the centroid is calculated by an iterative algorithm that minimizes the sum of the distances between each objects' centre and the cluster's centroid. This is done until the sum cannot be decreased further. Further details are explained in Chapter 4.

2.5.3. Trajectory magnitude normalization by z-score

The trajectory signals generated by the cluster's centroid where normalized by z-score. The z-score technique is very useful for standardizing data because it allows the comparison of measures with different scales, for example in classifications of Neardental bones (Gómez-Olivencia et al., 2015), human heart signals (Graff et al., 2011) or multivariate sequences (Richardson et al., 2010). Z-Score is a statistical measurement of a score's relationship to the mean in a group of scores. A z-score of 0 means that the score is the same as the mean. A z-score can be positive or negative, indicating that it is above or below the mean and by how many standard deviations. In terms of the standard deviation, the z-score measures the distance from a data point to the mean. The resulting dataset has the same skewness and kurtosis than the original one because the data are standarized with zero mean, thus retaining all the original shape properties. For sample, for data with mean μ and standard deviation σ, the z-score of a certain data point x is:

$$z = \frac{(x - \mu)}{\sigma} \qquad (1)$$

2.5.4. Fractal dimension

Fractal analysis, applied to signal processing, has found a wide area of applications in a variety of scientific fields from medicine (Accardo et al., 1997; Cáceres et al., 2004; Spasic et al., 2011) to speech recognition (Ezeiza et al., 2013), walking pattern recognition (Sekine et al., 2002), stress indicators in goats (Alados et al., 1996) and in fish motion studies (Inada and Kawachi, 2002; Tikhonov and Malchow, 2003; Tikhonov et al., 2001). The Fractal Dimension (FD) of a system is one of the most significant features to describe its complexity. Fractal systems have a characteristic called self-similarity, i.e., a close-up examination of the system reveals that it is composed of smaller versions of itself (Figure 2.1). Self-similarity can be quantified as a relative measure of the number of basic building blocks that form a pattern, and this measure is defined as the fractal dimension, which is rarely an integer number. Usually, the more complex the signal, the higher its fractal dimension value (Kith et al., 2009). Fractal dimension analysis has been successfully applied by López-de-Ipiña et al. (2013) to speech analysis and by Nimkerdphol and Nakagawa (2008) to show quantitative differences in the swimming behaviour of zebrafish (*Danio rerio*) provoked by the presence of hypochlorite in the water.

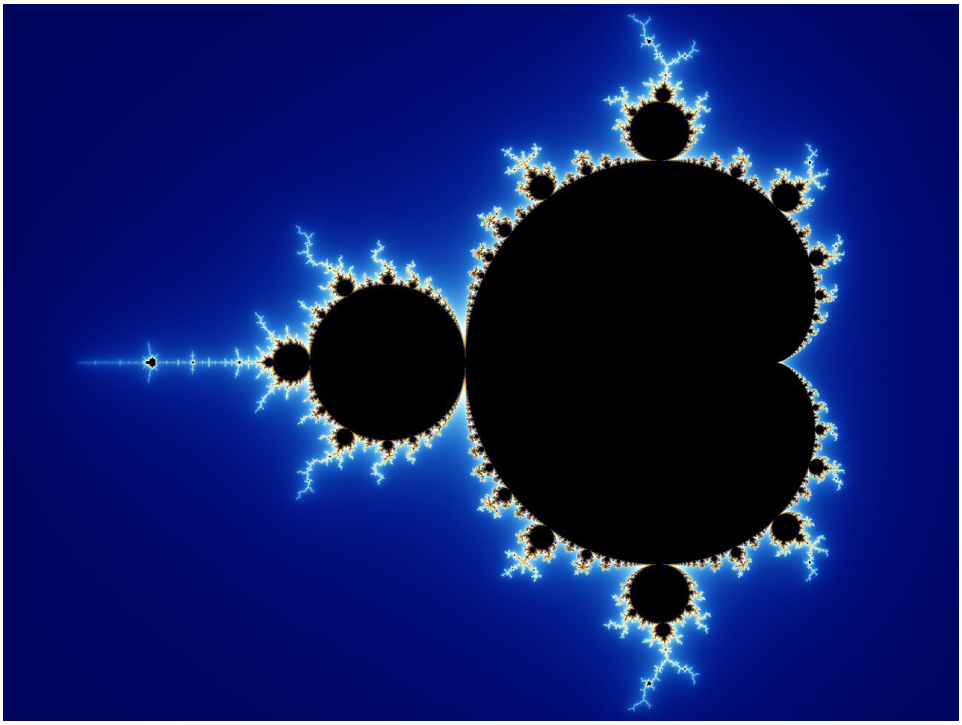


Figure 2.1. Initial image of a Mandelbrot set. Often used as an example of what a fractal is, Mandelbrot's set's shape adds smaller versions of the main shape. This characteristic is called self-similarity and it is applied to the entire set, and not just to its parts. "Mandel zoom 00 Mandelbrot set". Licensed under CC BY-SA 3.0 via Commons - https://commons.wikimedia.org/wiki/File:Mandel_zoom_00_mandelbrot_set.jpg#/media/File:Mandel_zoom_00_mandelbrot_set.jpg

Among the various algorithms available to measure the fractal dimension, we selected those specially suited to time series analyses that do not need previous modelling of the system. Two of those algorithms are Higuchi (1988) and Katz (1988) named after their authors. We used the former as the main reference, and a modification proposed by Castiglioni (2010) on the original version developed by Katz (1988). Higuchi was our first choice because it has been reported to be more accurate (Tsonis, 2007) but in most of these studies, the algorithm compared to was the original one developed by Katz himself. Castiglioni's improvement is theoretically sound and has not been tested in many experiments, so we considered interesting to test it as an alternative.

The algorithm proposed by Higuchi (1988) measures the fractal dimension of discrete time sequences directly from the time series $\{x_1,x_2...x_n\}$. The algorithm calculates the length $L_m(k)$ for each value of m and k, where m is the initial time $\{m=1,2...k\}$ and k is the time interval $\{k=1,2...k_{max}\}$. N is the length of the sampled signal.

$$L_m(k) = \frac{\sum_{i=1}^{\frac{N-m}{k}} |x(m+i_k) - x(m+(i-1)k)|(n-1)}{\left\lfloor \frac{N-m}{k} \right\rfloor k} \quad (2)$$

After that, the sum of all the lengths $L_m(k)$ for each k is determined by:

$$L(k) = \sum_{m=1}^{k} L_m(k) \quad (3)$$

And finally, the slope of the curve ln(L(k))/ln(1/k) is estimated using the best fit by linear least squares. The result is the Higuchi fractal dimension.

On the other hand, Katz (1988) proposed a normalized formula of the fractal dimension:

$$FD = \frac{\log(n)}{\log(n) + \log(d/L)} \quad (4)$$

Where the length L and the extension d of the curve are normalized using the average step a=L/n using (5) and (6).

$$L = \sum_{i=1}^{n} l_{i,i+1} \quad (5)$$

$$d = \max\{l_{i,j}\} \quad (6)$$

Nevertheless, given that the input signal is a mono-dimensional waveform, the length and the extension can be rewritten using Mandelbrot's approach (Mandelbrot, 1965). A simple and efficient way to do this is directing these two magnitudes in their own dimension as it was done by Castiglioni (2010). One by one, the extension on the Y axis is the range of y_k as seen in:

$$d = \max\{y_k\} - \min\{y_k\} \quad (7)$$

And the length L is the sum of all the increments in modulus as in:

$$L = \sum_{k=1}^{n} |y_{k+1} - y_k| \qquad (8)$$

This latter calculation is the Castiglioni's variation of Katz's algorithm (Castiglioni, 2010).

Once the fractal dimension algorithms were selected, it was extremely important to choose the window size to be used for the calculations. The fractal window goes through the signal, i.e. the object to analyse. This sliding window has a fixed size during each analysing period. In order to calculate the fractal dimension's features of the signal there are no restrictions other than the total waveform length to the window size to be used. Each signal was analysed using three fixed, but configurable, window lengths: 320 points, 640 points and 1,280 points. The third window size of 1,280 points was tested because previous studies that take into account the window size of similar dimension estimations suggest that a bigger window could be useful in some cases (Esteller et al., 2001; Tsonis, 2007). Since the fractal dimension is a tool intended to capture the dynamics of the system, with a short window the estimation would be very local and adapting fast to the changes in the waveform. When the window is longer, some details will be lost but the fractal dimension anticipates better the characteristics of the signal (Ezeiza et al., 2013).

2.5.5. Entropy

Conceptually, and as part of thermodynamics, the entropy describes how a system answers to changes in the surrounding environment and indicates the energy balance dispersed by the system itself: the higher the entropy, the higher the energy balance. Although it is not widely accepted, it can be said that entropy is a measure of disorder in a physical system.

Applied to biology, the entropy of a system, as a nonlinear measurement, has found application in complex biological systems and has occasionally been decisive to understand the nonlinear nature of a problem. For instance, Kulish et al. (2005) analysed brain activity using the spectra of the fractal dimension based on the Renyi entropy: combined with a visualization tool, these authors showed an intrinsic asymmetry of the brain activity. Permutation entropy has been used in a wide range of applications where measurements of complex time series were needed. As an example, Li eta al.(2008) measured the effects of sevofluore on the complexity of electroencephalographic series, Liu, Chon et al. (2011) analysed the movement of the fruit fly and Brandt and Pompe (2002) studied the complexity of chaotic time series.

2.5.5.1. Shannon entropy

Theoretically, the Shannon entropy (SE), as proposed by Shannon on his studies on languages (Shannon, 1948), allows the estimation of the average minimum number of bits needed to encode a string of symbols based on the alphabet size and the frequency of the symbols. This shows the minimal number of bits per symbol needed to encode the information in binary form, in case the logarithm base is 2. Shannon used this entropy measurement to estimate redundancy in the English language (Shannon, 1951). Formally, the entropy H(X) of a single discrete random variable X is a measure of its average uncertainty. The Shannon entropy (Shannon, 1948) is calculated by the equation:

$$H(X) = -\sum_{x_i \in \Theta} p(x_i) \log p(x_i) = -E[\log p(x_i)] \qquad (9)$$

Where X represents a random variable with a set of values Θ and a probability mass function $p(x_i) = P_r\{X = x_i\}, x_i \in \Theta$, and E represents the expectation operator. Note that p logp=0 if p=0.

For a time series representing the output of a stochastic process, that is, an indexed sequence of random variables, $\{X_i\} = \{X_1 \ldots X_n\}$, with a set of values $\theta_1, \ldots, \theta_n$, respectively, and $X_i \epsilon \theta_i$, the joint entropy is defined by:

$$H_n = H(X_1 \ldots Xn) = -\sum_{x_i \in \theta} \ldots \sum_{x_n \in \theta_n} p(x_1 \ldots x_n) \log p(x_1 \ldots x_n) \qquad (10)$$

Where $p(x_1 \ldots x_n) = P\{X_1 = x_1 \ldots X_n = x_n\}$ is the joint probability for the n variables $X_1 \ldots X_n$.

By applying the chain rule to Equation (9), the joint entropy can be written as a sum of conditional entropies, each of which is a non-negative quantity:

$$H_n = \sum_{i=1}^{n} H(X_i | X_{i-1} \ldots X_1) \qquad (11)$$

Therefore, it can be concluded that the joint entropy is an increasing function of n. The rate at which the joint entropy grows with , i.e., the entropy rate h, is defined as:

$$h = \lim_{n \to \infty} \frac{H_n}{n} \qquad (12)$$

For stationary ergodic processes (random processes) the evaluation of the rate of entropy has proved to be a very useful parameter (Eckmann and Ruelle, 1985; Grassberger and Procaccia, 1983a, 1983b; Pincus, 1991; Shaw, 1981).

2.5.5.2. Permutation entropy

As Shannon entropy, permutation entropy quantifies the disorder of a system by measuring its energy balance. This algorithm has shown a nice ability to measure complexity in large time series. Basically, this method converts a time series into an ordinal pattern series where the order of relations between the present and a fixed number of equidistant past values at a give time are described (Bandt, 2005; Liu et al., 2011a).

Following this idea, Bandt and Pompe (2002) proposed a permutation entropy method based on the Shannon entropy measurement with the purpose of visualizing and quantifying changes in the time series. The permutation entropy is calculated for a given time series $\{x_1,x_2...x_n\}$ as a function of the scale factor s. In order to be able to compute the permutation of a new time vector X_j, $S_t=[X_t,X_{t+1}...X_{t+m-1}]$ is generated with the embedding dimension m and then arranged in an increasing order: $[X_{t+j_1-1}\leq X_{t+j_2-1}\leq\cdots\leq X_{t+j_n-1}]$. Given m different values, there will be m! possible patterns π, also known as permutations. If f(π) denotes its frequency in the time series, its relative frequency should be p(π)=f(π)/(L/s–m+1). The permutation entropy is then defined as:

$$PE = -\sum_{i=1}^{m!} p(\pi_i) \ln p(\pi_i) \qquad (13)$$

Summarising, permutation entropy refers to the local order structure of the time series, which can give a quantitative measure of complexity for dynamic time series. This calculation depends on the selection of the m parameter, which is strictly related with the length N of the analysed signal. For example Bandt and Pompe (2002) suggested the use of m=3...7 following always the rule of:

$$m! < N \qquad (14)$$

If m is too small (smaller than 3) the algorithm will work wrongly because it will only have few distinct states for recording. When using long signals, a large value of m is preferable but it would require a larger computational time.

In our particular case and due to the computational cost derived from analysing signals composed from 5,000 samples, the m parameter was fixed at a steady value of m=4.

2.5.6. Descriptive statistics. Mean and standard deviation

Prior to data analysis, the data were subject to a Shapiro Wilk test to study the type of distribution they followed. The data generated by the experimental cases followed a normal distribution. Thus, they were analysed using descriptive statistics for measuring data dispersion and location: arithmetic mean and standard deviation.

- Arithmetic mean refers to the central value of a discrete set of values in a given dataset. The arithmetic mean is calculated by:

$$\mu = \frac{1}{N}\sum_{i=1}^{N} x_i \qquad (15)$$

Where μ is the mean of the data, N is number of scalar observations and x is each given data value.

The standard deviation measures the dispersion of a data set. The one used in this work, uses the Bessel correction due to the fact that it is applied to samples instead of to populations. It is computed by:

$$\sigma = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \mu)^2} \qquad (16)$$

Where σ is the standar deviation, μ is the mean of the data, N is number of scalar observations and x is each given data value.

2.5.7. Boxplots

Boxplot is a graphical representation of the data distribution. The data is represented in a box where the central red line inside the box is the median of the data. The upper and lower lines represent the 75^{th} and 25^{th} percentile of the sample data respectively and the distance between those lines is the inter-quartile range. The whiskers (black lines) extend to the most extreme data points not considered outliers, while outliers (indicated by red plus signs) are data points whose value is outside 1.5 times the inter-quartile range.

2.5.8. Curve fitting and goodness of the fit

Graphical curve fitting allows the linear or non-linear regression of data in order to calculate a model that fits the original data. Graphical fitting offers, in addition, the possibility of viewing the entire dataset at once and the possibility to display a wide range of relationship between the newly calculated model and the original data.

The model can be obtained by different linear and non-linear approaches such as different grade polynomials, exponentials, Gaussians, etc. Once the model is calculated, a series of parameters are used [sum of squares due to error (SSE), R-square, adjusted R-square and root mean squared error (RMSE)] to estimate the goodness of the fit. A brief description of each is:

- Sum of squares due to error (SSE) compares the total difference between the original values and the response values from the fit. The closer to 0 the better the fit.
- R-square is measured between 0 and 1, where a closer value to 1 indicates that the model explains a greater portion of the variance. It explains the success of the fit in relation to the variation of the data.
- Adjusted R-square is based in the previous R-square measure. This adjustment is based on the residual degrees of freedom derived from the relationship between the number of response values minus the number of fitted coefficients estimated from the response values. Again, it is defined between 0 and 1 and a closer value to 1 indicates a better fit.
- Root mean squared error (RMSE) is the standard error of the regression and it estimates the standard deviation of the random component in the data. A closer value to 0 indicates a better fit.

In the present Thesis two types of fitting approaches are used due to the non-linear nature of the data obtained.

- By using a two term grade power fitting adjusted by the Non-Linear Least Squares method with Trust Region algorithm, which obeys:

$$y = u \cdot x^v + w \qquad (17)$$

And where u, v, w are the fitted parameters and x is the input variable and y defines the output model.

- A first order polynomial fitting, adjusted by Linear Least Squares method, which obeys:

$$y = u \cdot x + v \qquad (18)$$

Where u and v are the fitted parameters, x is the input variable and y defines the output model.

2.5.9. Modelling

Conceptually, control engineering has to deal with three types of problem depending on the known data. As already mentioned in Chapter 1 a system can be depicted as shown in Figure 2.2, as inputs (u), outputs (y) and their relation defined by the system (S).

The three types of problems are summarized in Table 2.1, and the strategy to face the problem depends on the variables known.

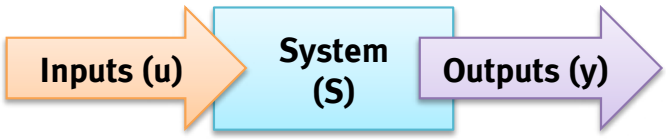


Figure 2.2. Basic system. A basic model is created from the interaction between the inputs and the outputs of a system.

Table 2.1. Different type of systems engineering problems. Depending on the knowns and unknowns, there can be defined three types of problems.

Knowns	Unknowns	Problem definition
u, S	y	Simulation
u, y	S	System Identification - Modelling
S, y	u	Control

The present Thesis deals with a Systems Identification or Modelling problem where only the inputs and outputs of the system are known. There are, subsequently, different methods to face it, depending on the prior knowledge of the system (Ljung, 2001). The basic rule for system identification should be not to estimate information that is already known. This leads into three types of model depending on that previous knowledge:

- White box models. The model therein is perfectly known and it is possible to build it from prior knowledge. Often through physical equations.
- Black box models. No physical knowledge is available about the system and it is calculated from observed data.
- Grey box models. A mixture of both cases, where some prior knowledge is known but other parameters need to be estimated from observation.

This Thesis uses the black box modelling technique due to the lack of previous knowledge about the system. Thus, the data input-output relationship is established and the model built based on the experimental cases performed in the present work and explained further on (Chapters 4-6).

Black box modelling is a widely used technique for approximating very complex and/or unknown systems. Its application range is very wide and goes from feedback controller design systems with time constrains (Golubev and Horowitz, 1982), to cancer biomarker identification (Kalaitzakis et al., 2008) or noise source identification in an electric machine (Ma and Zuo, 2014).

The proposed model is built according to the block diagram principles. Block diagram is a way of representing a model in a graphic manner, which allows the understanding of the relationship between the variables and the subsystems therein. The block diagram representation can be defined as the graphic representation of a equation system (Cellier, 2013; Matko et al., 1992; Ogata, 2009; Recktenwald, 2000; Woods and Lawrence, 1997). Basically, the components of this graphic representation are transfer function blocks, arithmetic operation nodes, bifurcation nodes and interconnection arrows (Figure 2.3).

- A transfer function block characterizes the relationship between the input-output variables linked (Figure 2.2).
- An arithmetic operation node is able to perform a sum or a rest between all the signals converging.
- A bifurcation node is a point where a signal is diverted to a different destination. It allows multiple destinations.
- An interconnection arrow joins the signals from the different components in the block diagram. The sense of the signal is the sense of the arrow.

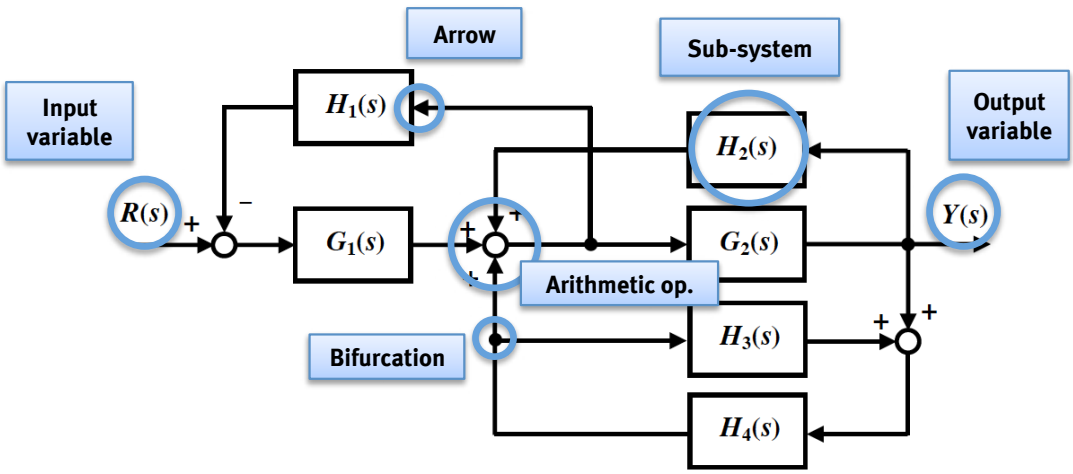


Figure 2.3. A block diagram representation of a system. Blocks are connected by arrows, and interconnection nodes and bifurcations establish the relationship between the blocks. Example from Ogata (2009).

2.5.10. *Programming*

Data processing, equation implementation, analysis and the design of the model have been performed using Matlab 2014a. Scripts and programs are available upon request.

2.6. Limitations of the study

The limitations of the present study are defined by those characteristics of its design and/or methodology that have influenced the application, interpretation or generalization of the results. From the practical point of view, the present study presents the development of an engineering tool and its application. Engineering, as any applied science, manages the finding of the best solution to a problem at an assumable load and/or cost. Thus, if reaching an optimal solution is not viable, a suboptimal one must been found which fulfils all the requirements needed. Being consequent with this principle and following the philosophy of systems engineering, where a big problem is divided into smaller and more easily solved ones. The present study presents a series of small interconnected problems where the overall output can be improved by improving the individual components. In other words, adding marginal gains, a mayor gain can be achieved.

2.6.1. *Limitations of the research methods used*

Data acquisition has been performed using one single camera per tank inside seawater. Some of the limitations this fact imposes on the work are:

- Fish move inside the tank in a three-dimensional environment but video recording is made in two dimensions. Therefore, data processing is made based on the two-dimension signal.
- The underwater working environment itself creates a number of problems related mostly to the image-processing step. This means that a robust algorithm focused on generalization was required to work satisfactorily in most of the circumstances, although it might lose accuracy when a very precise measurement is needed. Thus, an algorithm was created to deal with this rough, harsh and continuously changing environment, in order to make it stable from an operational point of view. On the other hand, any algorithm designed for a specific operational point may not work well under a wider range of conditions.

Thus, all the algorithms used and steps followed in the present Thesis have been selected according to their suitability, robustness and ability to lead to measurable and comparable results, at a reasonable computational cost. Every step made from optical flow generation to centroid trajectory analysis may be improved.

2.6.2. Limitations of the case studies

Sceptics about case study methodology argue that the study of a small number of cases does not offer any grounds for generalization of the findings. Nevertheless, the case study method is widely used in science and it is still one of the most successful to study real-life situations if the cases therein are carefully planned and performed.

One of the key limitations in this work belongs to the use of a specific animal model, which has intrinsic characteristics. Analysing the entire animal response means that one must deal with:

- Species-specific attributes and behaviour.
- Ability to camouflage inside the tank.
- Circadian circles.
- Acclimation periods need to avoid non-desired stress factors.
- Individual variability may be larger than variability between different species.
- Others.

Another key limitation regarding the case study method belongs to the experimental design. In most cases, the experimental design must take into account a mixture of factors such as physical space, time, cost, experimental subject availability, etc. As already mentioned, the case studies presented in this work are subject to improvement; nevertheless, the results obtained have been analysed taking into account the experimental design and assessed within the imposed limits.

2.6.3. Limitations of the model

Two main factors affect the model proposed by this Thesis: the selected input-output variables and the data used.

The input-output variables of the proposed model where the fish number, as input, and the Shannon entropies of the basal state and of the event response, as output. Moreover, an additional model relates the Shannon entropy of the basal state as input and that of the event response as output. This decision was made in order to avoid using the fish trajectory as a variable for the model due to the fact that it is a noisy, patternless and unpredictable signal.

Regarding the second point, the data used were obtained from two experimental cases. Most of the data come from the experimental case described in Chapter 5, although some data from Chapter 6 are also used. Although the experimental conditions are not identical in both cases, the data were used as if they belonged to the same experiment.

CHAPTER 3
STATE OF THE ART

The present Chapter deals with the state of the art in the use of Biological Warning Systems using fish as the sensor and its application to the aquaculture industry. The last part of this chapter includes a brief explanation about the application to fish monitoring of some of the specific research methods described in Chapter 2.

Probably one of the best known Biological Warning Systems is the canary bird used in coal mines to detect toxic gases: display of distress signs by the birds was an indication of the presence of any of a number of toxic fumes and the miners knew they had to abandon the area immediately. Nowadays there is a great interest in developing suitable Biological Warning Systems to monitor drinking water, in part due to fears of terrorist attacks (Environmental Protection Agency, 2005; Hasan et al., 2004) but also, as mentioned in Chapter 1, because of the large and increasing number of novel substances that find their way into the food and water supply.

The use of fish behaviour as an indicator of a perturbation is not completely new. The use of fish as Biological Warning System to monitor water quality started in the 1970's and has been reviewed by van der Schalie et al. (2001) and by Bae and Park (2014). It often consists of observing, and optimally quantifying, the behaviour of model fish species placed in the water whose quality needs to be monitored. Changes in fish behaviour suitable to serve as indicators of developing stressful conditions include: alterations in motion pattern, loss of positive rheotaxis, changes in ventilatory responses (ventilatory rate, depth, amplitude) and coughing (van der Schalie et al. 2001 and references therein). Hypoxia, feeding regime and pH alter the behaviour of fish (Nimkerdphol and Nakagawa, 2008; Polonschii and Gheorghiu, 2013) as does, in a quantitative manner, exposition to methylmercury (Eguiraun et al., 2015b, 2014). Exposure of young minnows to antidepressants made them become anxious, anti-social and sometimes even homicidal, apparently by altering neuronal development (Brodin et al., 2013). High fish density is also a perturbation factor to take into account (Di Marco et al., 2008; Papoutsoglou et al., 1998). These studies showed that introduced perturbations altered fish behaviour and that the responses were indeed quantifiable. This fact opens the possibility of using fish behaviour itself as a biomarker for environmental monitoring (Kalogerakis et al., 2015) and in aquaculture settings (Eguiraun et al., 2015a), appearing to be better suited than, or at least complementary to, chemical analyses, in order to estimate the welfare of fish and the potential toxicity of seafood (Chapter 1). For example, in the case of selenium/mercury mentioned in Chapters 1 and 6, the chemical analysis alone of the levels of each metal would not provide a suitable answer regarding the health of the system, lacking the integrated response that is one of the characteristic for such a system. Unknown substances will not be tested for.

Commercially available products and methods measuring behavioural changes of fish swimming in flowing water were revised by Mons et al. (2008). But currently, there are no suitable on-line non-invasive methods to monitor fish welfare; and the presence of contaminants must be detected by analysing the feed and the water, usually by analytical chemistry methods, which are expensive and may take several days from the moment the sample is taken, until the results reach the farmer. As this Thesis does, Hellou proposed the introduction of the concept of Biological

Warning System (BWS) into aquaculture by using the fish themselves as the sensor (Eguiraun et al., 2015a; Hellou, 2011).

The works on the implementation of Biological Warning Systems for water quality monitoring use a model species that is placed in a tank with the water to be monitored. However, in an industrial aquaculture setting and given that the feed is at least as likely to be the source of contaminants as the water (and/or of the lack of essential nutrients, which will also cause stress and poor quality), we believe that a Biological Warning Systems should be developed for each farmed species using the farmed specimens and conditions (species, genetic stock, feeds, veterinary treatments and materials in contact with the fish) as the warning system. One of key issues to decide is the method for monitoring data acquisition: images are very common (Bae and Park, 2014; Eguiraun et al., 2014), but also the use of ultrasounds (Føre et al., 2009; Polonschii and Gheorghiu, 2013; Polonschii et al., 2013) and infrared light (Pautsina et al., 2015) have also been proposed. Invasive data acquisition methods such as the placing of electronic transmitters (Føre et al., 2009), electrodes or wires on or in the fish [see review by Bae & Park (2014)] should not be considered because they are not compatible with the welfare of the fish.

As already mentioned in Chapter 1, another key issue in developing a Biological Warning System is the choice of the method to treat complex information. Non-linear processing, and particularly entropy and fractal dimension techniques, have become very promising tools for measuring the complexity of the trajectories of apparently random search paths in animal behavioural studies. For example, the characteristics of a 2-dimensional path of moving animals measured using their fractal dimension will give a value between 1 (for a perfect straight path) and 2 (for a fully jagged and wiggly path) (Bae and Park, 2014; Benhamou, 2004; Etzenhouser et al., 1998). Other useful algorithm is the Fourier transform, an operation that reversibly transforms a complex-valued function of a real variable in time or space into a new function of frequency. A two-dimensional Fast Fourier Transform was used by Park et al. (2005) to examine changes in the behaviours of medaka (*Oryzias latipes*) before and after treatment with 0.1 mg/L of diazinon, an organophosphate insecticide. The x and y coordinates of the medaka in the tank were continuously recorded in two dimensions with a digital image processing system and used to calculate the two-dimensional Fast Fourier Transform which was able to reveal differences in the movement of the medaka before and after the treatment. Other authors have successfully applied self-organizing maps and a hidden Markov model to detecting differences in the behaviour of zebrafish (*Danio rerio*) as a response to the presence of formaldehyde in the water (Liu et al., 2011b).

Other studies aim at the construction of machine-based systems for fish disease diagnosis. The main challenge in those works is to develop the knowledge database, a task which is time and resource consuming and focuses mainly on water quality (Ma et al., 2010) and/or on nutritional problems, parasites, viruses, bacteria and fungal agent diseases (Li et al., 2002). Other investigations aim at developing early warning systems (Li et al., 2009), including the use of sms alert procedures (Miaojun et al., 2013).The main challenge for these fish-health oriented

studies is usually the development of the decision component rather than the warning systems itself.

To summarize, Biological Warning Systems have found wide application in the monitoring of water quality although full self-controlling systems have not been developed yet. It is also generally agreed that this methodology must be used together with biosensors and with analytical chemistry for a finer identification of the contaminants and stressors: i.e., the current monitoring methods (analyses of feeds, water, materials, etc) should continue to be in place in order to prevent contaminants enter the food chain. The shift we propose to introduce concerns the standard procedure of working itself, i.e. the paradigm shift consists in the implementation of Biological Warning System as an on-line continuous monitoring practice to verify 1) the integrity of the system, 2) the absence of unknown harmful substances and 3) to non-invasively monitor health, quality and welfare. Thus, the Biological Warning System would be the - new - first signal to be received by the monitoring sub-system (rather than wait several hours or days to get the answers from analytical methods) and should be a core part of the Hazard Analysis and Critical Control Point.

CHAPTER 4

APPLICATION OF ENTROPY AND FRACTAL DIMENSION ANALYSES TO THE PATTERN RECOGNITION OF CONTAMINATED FISH RESPONSES IN AQUACULTURE

4.1. Introduction

As already mentioned, biological systems, such as a group of animals, are regulated by interacting mechanisms that operate across multiple spatial and temporal scales. When studying such a biological system, we are interested, as defined by Kitano (2002), on how the large numbers of functionally diverse and multifunctional set of elements (i.e. the individual fish in the present work) interact selectively and nonlinearly to produce coherent rather than complex behaviours.

The objective of this chapter is to develop a tool based on image acquisition, which should be used to on-line monitor fish welfare. The tool presented here uses signal processing and nonlinear trajectory analysis of the collective fish response to a stochastic event as the unit to measure. Video recording was chosen for its simplicity and low cost and fractal dimension and entropy for their proven suitability to identify nonlinear features (see Chapter 1).

The aim the Thesis is not focused on fish behaviour, which is a very complex and species-specific attribute (Magnhagen et al., 2008). It is focused on the response to a stochastic event, which is simpler to measure and requires less computational effort. Following Kitano's idea (Kitano, 2002), the work analyses the coherent response of the group rather than the individual response of each fish, which in addition to requiring much more computational effort it may be impractical in real-life settings where there may be several thousands of individuals in the same area or cage. That is also the reason why we believe that this methodology, focused on the group's response to an event, is more suitable to be generalized and applied to other species and experimental settings.

The tool was tested in the experimental cases shown in Chapters 4 and 5, two of them with no exposure to any toxic agent, and a third one that was exposed to a neurotoxic agent called methylmercury. As already explained in Chapter 1, methylmercury was selected because of its increasing relevance as an environmentally ubiquitous pollutant that accumulates and biomagnifies in the trophic chain (FAO, 2010).

4.2. Materials and Methods

4.2.1. Experimental cases

Three experimental cases were used to test the tool: C_1 and C_2, consisting of 81 fish each and differing only in that C_2 fish were tagged with Visual Implant Elastomer by Northwest Marine Technology (Brennan et al., 2005) and C_3 with 41 fish that had been treated for 9 days with 4 µg methylmercury chloride/L according to Branco (2012). Methylmercury chloride (CH_3ClHg) had been purchased from Sigma-Aldrich product number 33368.

During all the experimental period, the fish were subjected to a 12h /12h dark/light photoperiod and they were fed once a day INICIO Plus feed from BioMar (56% crude protein, 18% crude fat). The fish were placed in tanks (100 cm x 100 cm x 90 cm) filled up to 80.5 cm of height with 810 L of aerated seawater under direct light (2 x 58 W and 5200 lm) avoiding shadows as much as possible. To record the fish response one camera was placed in each tank positioned exactly in the same place.

4.2.2. Image acquisition and processing

The schematic diagram of the working procedure is described in Figure 4.1. The video sequences and images were acquired using a GoPro Hero3 high definition camera in its GoPro Underwater housing attached to the tank by GoPro Side and Flat mounts placed in the top right corner of the tank: 3.8 cm from the right wall, 15 cm from the top of the tank and 5.5 cm below the water level. The GoPro Hero3 high definition camera was selected for convenience: its size is small, thus minimizing the effect of introducing foreign objects in the tank, and it has a water-proof protective case very convenient because it has to be submerged in contaminated water, so that at the end of the experiment the camera can be reused while the case is discarded as contaminated material. The recordings were made in high definition RGB (Red Green Blue) scheme at 1440p, 24 frames per second (fps) and in 4:3 picture size. A SanDisk 32 Gb Ultra microSDHC™ (Class 10) secure card was used for the recording and a 2 Tb Hard Disk for storing the data. In order to minimize stressing factors, continuous recordings were done until the batteries of the cameras run out, which took about 1 h and 30 min. Within this period, a stochastic event consisting of a sudden hit in the tank was introduced and the 30 s pre- and 3 min post-event were processed (Figure 4.2).

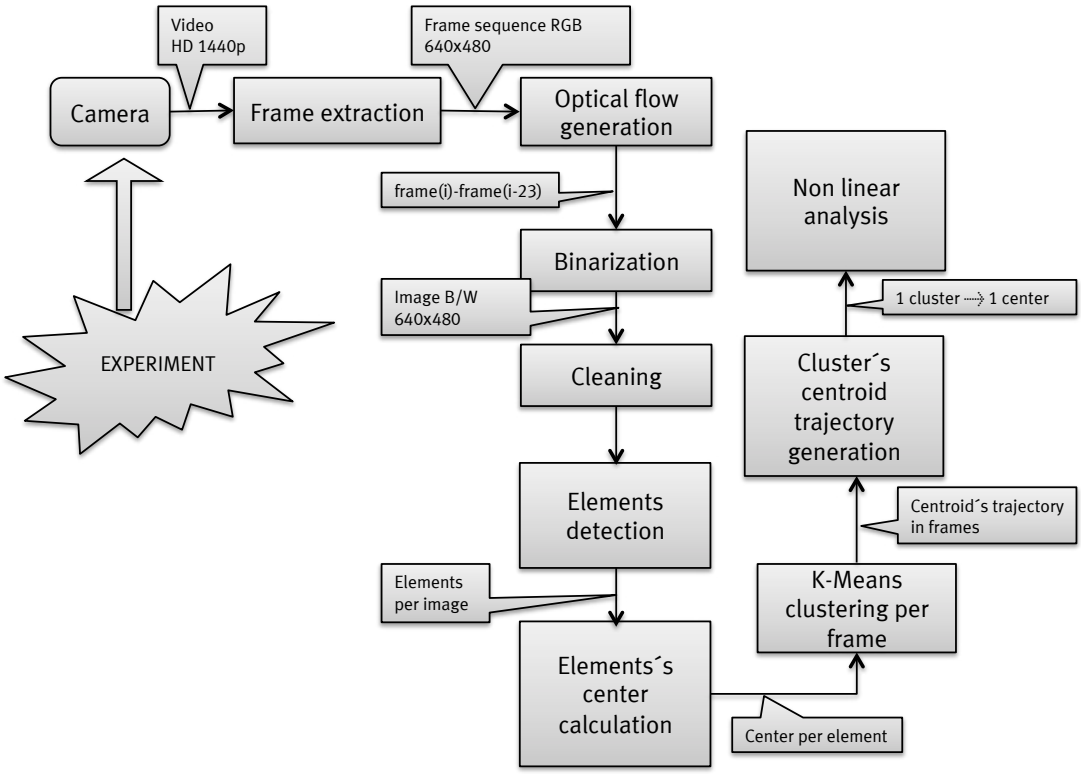


Figure 4.1. Schematic representation of the experiment's workflow showing the steps from image acquisition and processing to data treatment. In short, the video frames were converted to black and white images from which the center of each object was first calculated and used to estimate (by k-means clustering) the center of all the objects, or centroid. Then, the trajectory of the centroid over all the video sequence was calculated. Finally, the trajectory of the centroid was treated by non-linear signal processing algorithms.

The 3 min 30 s of interest were located using the sound clip of each video analysed with Audacy free software to determine exactly where the event happened. The 3 min 30 s clip was cut from the main video and converted into a sequence of images. Since the video was recorded at 24 fps, it was converted also to 24 fps. The images were compressed from the 1440p HD format to the more convenient 640 pixel x 480 pixel format. Frame extraction and format conversion were made with the commercial iMovie software. After the video was converted into a colour image sequence, the background and noises were eliminated and it was then converted to black and white.

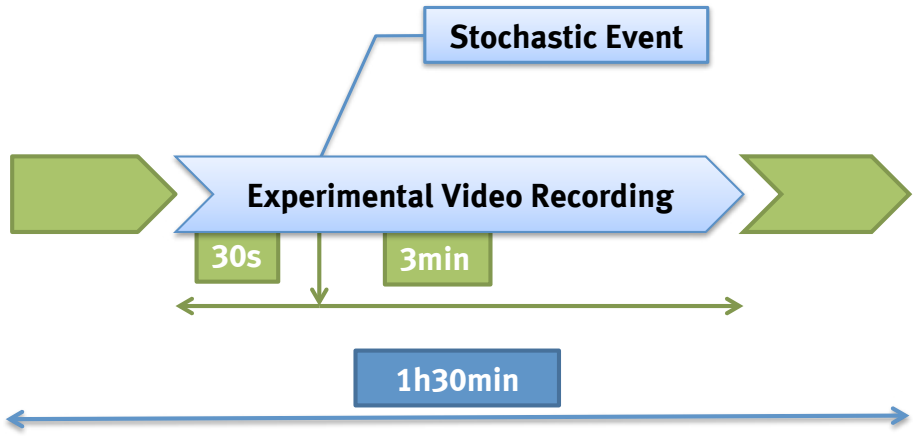


Figure 4.2. Recording procedure. The total recording time was 1h 30 min. The results presented in this Chapter correspond to the analysis the 30 s pre- and 3 min post-event.

4.2.3. Object detection and motion estimation

From the point of view of image segmentation and object detection, and due to the nature of the set up, a biological experiment in a real, small-scale environment, there were three main problems: noise, artefacts and occlusions.

Two main sources of noise were identified: air bubbles and shadows. The main noise was generated by the air bubbles moving towards the surface. This creates a little wave system on the water surface, which makes the light penetrate the water in a nonlinear manner. The second source of noise was the shadows of the fish on the bottom and the walls of the tank. Although the lighting was placed on the ceiling above the tank to avoid this issue, the generation of some shadows was unavoidable (see Figure 4.3).

There were three main types of artefacts (anomalies introduced in the signal or in the data by the equipment or the technique): the first was caused by the pellets used for feeding the fish. Some feed pellets were suspended in the water and when fish swam around them, they spread off forming black holes in the images (see Figure 4.3). The second (very similar to the first but smaller in size) was caused by the faeces of the fish, and the third by the light's reflections on the skin of the fish.

Figure 4.3. Example of an artefact indicated by the arrow, caused by a feed pellet. Occasionally, the system also had difficulties to discriminate some fish from their shadows (circle).

Occlusions are a well-known issue in tracking that occur when two or more tracked target images become one during a time period in the sequence. Occlusions are more frequent when target objects are similar to each other, as it happens in animal groups and fish shoals (Butail et al., 2012). Occlusions in fish tracking may lead to two types of misidentification: loss of fish identity and swapping identity between individuals (Delcourt et al., 2009). Tracking problems take place both while the occlusion occurs and when the occlusion ends and the fish appear separately in the image. Different solutions have been developed to solve this problem, such as the use of 3D information (Isard and MacCormick, 2001; Zhao and Nevatia, 2004), using the animals' characteristics, such as their shape (Branson and Belongie, 2005; Isard and Blake, 1996; Maccormick and Blake, 2000) or analysing the special topology of the shape (Khan et al., 2006; Rasmussen and Hager, 2001; Sanchez and Dibos, 2004; Sigal et al., 2004). Automatic scene calibrating systems are also very helpful tools and many approaches have been made in this field, for example an automatic calibrating camera system for tracking people (Perdomo et al., 2013).

Given that the robustness of the system depends on how the motion detection takes place and all the problems listed above severely limit the election of the motion estimation algorithm, we decided to use an algorithm based on optical flow in order to eliminate noise, artefacts and occlusions in one step. This conventional approach is based on the calculation of the local relative motion (Barron et al., 1992) which is also used for space segmentation (Ranchin and Dibos, 2004), and it has the additional advantage that it can work with a moving camera and/or with moving backgrounds. We applied this method to detect objects and estimate their motion by a simple process which consisted in identifying the differences between one of the images and the image obtained in the previous second, i.e., resting each frame from its 24^{th} predecessor (since we work at 24 fps). This made it possible to delete background, noise and artefacts common to all images, including objects that had not changed position in the previous second while keeping those objects whose position changes

in 1 second intervals, i.e., the moving fish. Methods based on optical flow provide very valuable information but they are computationally intense and sometimes require specific hardware.

After the optical flow, or motion, was calculated, the images were binarized using standard morphologic operations in order to be able to detect the elements in the image and their centers in each frame

4.2.4. Clustering and trajectory generation

In order to work in the most reliable way possible, knowing that our system, experiment and conditions have limitations, and being particularly concerned about a potential loss of information due to the image segmentation and processing methods, we decided to use a clustering method to identify the fish group and calculate the group's centroid.

The centroids' positions were estimated by k-means because this algorithm is robust, with a good relationship between speed and stability and it works well with large amounts of data. Thus, once the centers of the objects were calculated, and knowing their coordinates in the two axes within each frame, k-means was applied to find the center of the entire group. In our particular case, the dataset were the objects' centers in each frame, from the first frame to the last one.

Each cluster in the partition was defined by its member objects and by its centroid, or center: the point where the sum of distances from all the objects in the cluster is minimized. K-means computes the cluster's centroids differently for each distance measured in order to minimize the sum with respect to the distance specified. This is done using an iterative algorithm that minimizes the sum of the distances from each object to its cluster's centroid, over all the clusters. The algorithm moves objects between clusters until the sum cannot be decreased further. The result is a set of clusters that are as compact and separated as possible. It is possible to control the details of the minimization using several optional input parameters to k-means, including the initial values of the cluster centroids, and the maximum number of iterations. This algorithm, often applied for image segmentation, has successfully been used to detect animals (Nunes Goncalves et al., 2007). The centers of the clusters are calculated in a two-space dimension (2D), using both coordinates (X and Y) and the frame number, which goes from 1 to n in the image sequence, and then a trajectory table, is built (Table 4.1).

Table 4.1. The cluster's centroid coordinates are calculated for each frame and from them the trajectory of the cluster is estimated.

Frame number	X coordinate	Y coordinate	Centroid's coordinates
1	x_1	y_1	x_1,y_1
2	x_2	y_2	x_2,y_2
...	...	...	...
n	x_n	y_n	x_n,y_n

A figure (Figure 4.4) was then created by plotting the values obtained from Table 4.1: in the vertical axis the values corresponding to the pixel number of the x-coordinate of the centroid (in red) and to the pixel number of the y-coordinate of the centroid (in blue) and in the horizontal axis the frame number to which each x and y values correspond. Since the images have a 640 pixel x 480 pixel format, the scale of the vertical axis in Figure 4.4 goes from 0 to 640 pixel number (the lowest and highest theoretical possible values for the x-pixel number in Table 4.1, when the centroid is placed either in the right or in the left border of the frame). The lowest and highest theoretical possible values for the y-pixel number in the Table 4.1 are 0 and 480 respectively. The horizontal axis, representing the frame number varies in the three cases due to the processing of the video sequences. In all three cases there was a sharp change in the trajectory of the centroid in response to the stochastic event, which occurs around frame number 720. The panels to the right in Figure 4.4 show a magnification of the region of the plot corresponding to the response to the event.

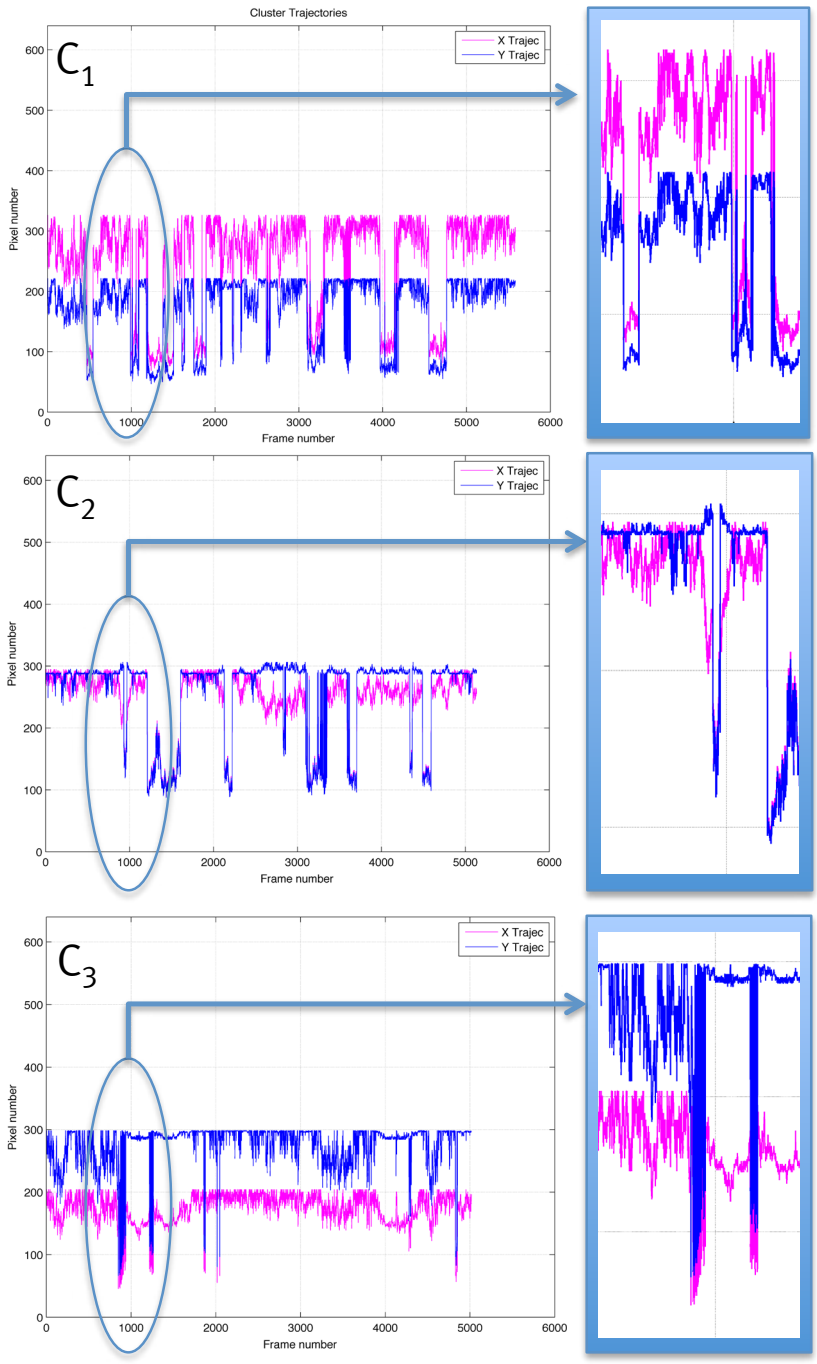


Figure 4.4. Plot of the values obtained from Table 4.1 for the three cases examined: C_1 (top), C_2 (middle) and C_3 (lower panel). The horizontal axis represents the frame number (from 0 to about 6,000 frames processed) and the vertical axis represents the pixel number in each frame for the x (red line, from 0 to 640) and y (blue line, from 0 to 480) coordinates of the centroid. A stochastic event took place around frame number 720, indicated by a circle and amplified in the right panel, which resulted in a sharp alteration of the centroids' trajectories in the three cases.

4.3. Results and Discussion

The non-invasive tool developed targeted the responses of the fish groups rather than that of individual fish, both to reduce the computational effort and because the response of the group may be considered the result of integrating all the responses contributed by each individual fish, while the latter may be influenced by the physiological status of the individual, its size, status in the school's hierarchy and other factors that are usually unknown when the monitoring is performed. Also, the response to a stochastic event was measured instead of other behavioural aspects (swimming pattern, daily activity, feeding, aggressiveness, etc.) because it permits to restrict the computational analysis in time to the duration of the response (three minutes in the present case, rather than observing the animals for longer periods of time when more variables may play a role) and to reproduce the event at will in other settings for comparison purposes. Longer periods of time were also analysed but the discriminatory power of the analyses did not improve (results not shown). Each of the three experimental cases, C_1, C_2 and C_3, were treated by fractal dimension and entropy algorithms. For fractal dimension analyses, Higuchi, Katz, and the Castiglioni's variation of Katz's algorithms were used with three window lengths of 320, 640 and 1,280 points per algorithm. While for entropy analyses, Shannon entropy and permutation entropy where calculated.

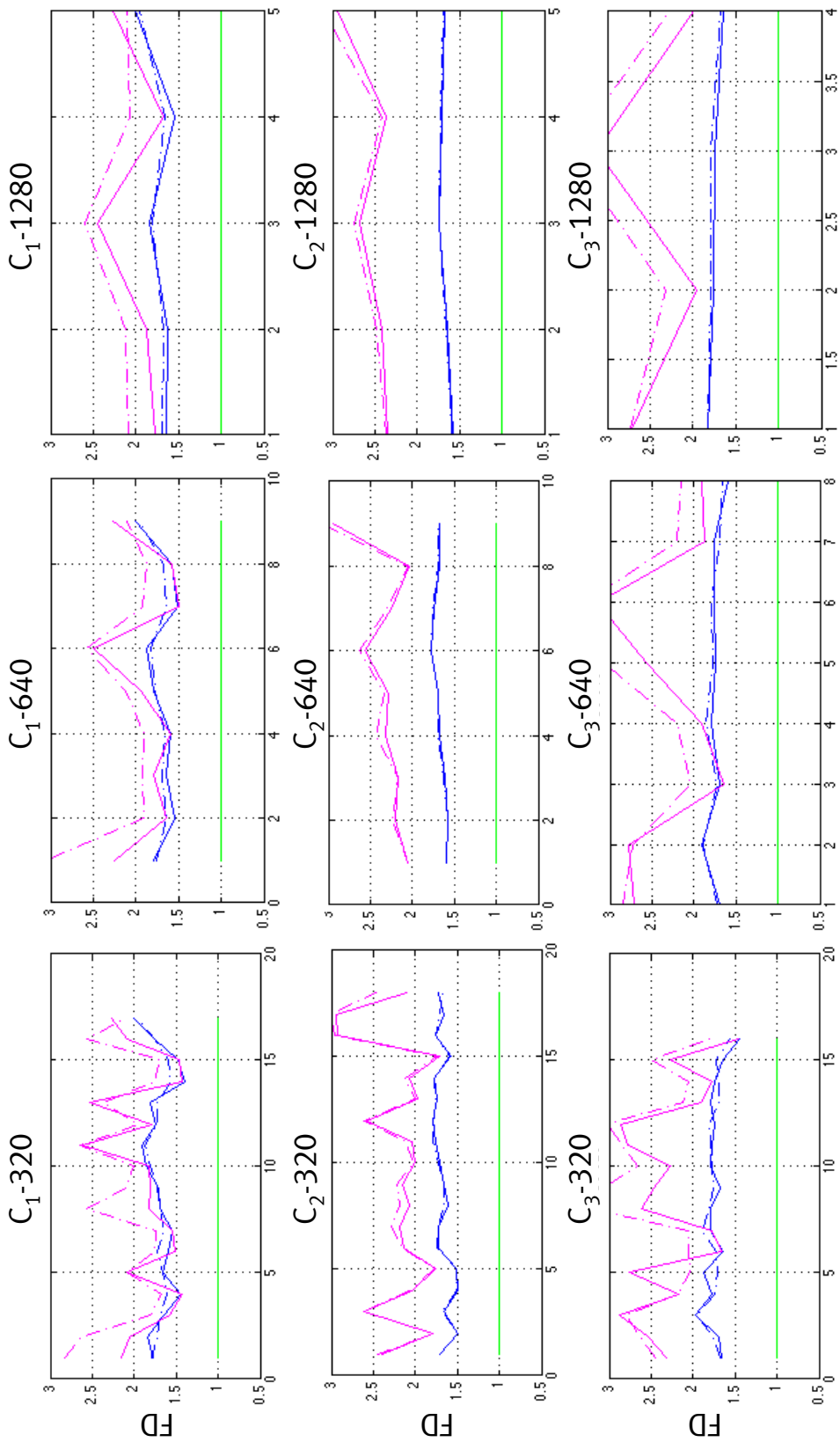
Generally, of the three cases examined, we expected C_1 and C_2 to behave similarly to each other and to be clearly different from C_3 mainly due to the methylmercury contamination in C_3 and partially due to the population variation. Nevertheless, based on previous studies (Di Marco et al., 2008; Papoutsoglou et al., 1998), it was not supposed to be expected a large difference attributable to the number of fish only, 81 fish (C_1 and C_2) versus 41 fish (C_3).

Two of the three fractal dimension algorithms used, Higuchi and Katz's variation proposed by Castiglioni were able to differentiate C_1, C_2 and particularly C_3, for the three sampling window lengths in both coordinates of the clusters' centers, X and Y in a two dimensional analysis, as shown in Figure 4.5. Since there was a correlation between the X and Y coordinates, the rest of the calculations were performed on only one of them, the X values. The almost constant, and close to 1, value of the fractal dimension obtained by using the Katz algorithm is in agreement with the results of Raghavendra and Narayana Dutt (2009) and confirms Castiglioni's note (Castiglioni, 2010). Indeed, the modification proposed by Castiglioni was more sensitive to detect differences between the three cases than Higuchi's algorithm, suggesting that for our particular application, the former may be the most suitable one.

The median and the standard deviation of the fractal dimensions obtained for each case and window length for the Katz-Castiglioni algorithm on the X values are plotted in Figure 4.6 and shown in Table 4.2. C_3 showed the highest fractal dimension median value for all three-window lengths with very similar values that were close to 2.5 (Table 4.2) but it also displayed the largest dispersion of values (Figure 4.6). C_1 had the smallest dispersion of values (Figure 4.6) and they had a tendency to increase with increasing window length. The fractal dimension of C_2 varied between 1.9 and

2.1. Increasing window length seemed to diminish the dispersion of the values in C_1 and C_2, but did not affect the degree of dispersion in C_3 (Figure 4.6). Interestingly, these results agree with those of Nimkerdphol and Nakagawa (2008) in spite of these authors using a different species (zebrafish, which is a freshwater species that prefers warmer water), only one individual and a different contaminant: the fractal dimension of the swimming trajectory of their zebrafish also trended to increase with increasing concentrations of sodium hypochlorite contaminating its water, obtaining fractal dimension values of the swimming trajectories between 2.11 and 2.14. The value of the fractal dimension varies depending on the algorithm used for its calculation, on the different units composing the time series, whether normalization has taken place or not as addressed by Fuss (2013), and on the window length; with lower values of window length producing lower fractal dimension values. Optimization of the window length for our particular case produced fractal dimension values higher than 2, which is also in accordance with the results of Nimkerdphol and Nakagawa (2008).

Figure 4.5. Comparison of the three fractal dimension algorithms (Higuchi, blue lines; Katz, green lines and Katz-Castiglioni, red lines) evolution for each case signal (X, broken lines; and Y, full lines) and for each window length (left column 320, middle column 640 and right column 1,280) for C_1 (top row), C_2 (middle row) and C_3 (lower row). In each graphic the vertical 0Y axis is the fractal dimension of the cluster's centroid and the horizontal 0X axis is the evolution of the fractal dimension per sliding window length. As mentioned in the text, the fractal dimension for the Katz algorithm gave a value of 1 regardless of the signal case or the window length.


C1-1280
C2-1280
C3-1280
C1-640
C2-640
C3-640
C1-320
C2-320
C3-320
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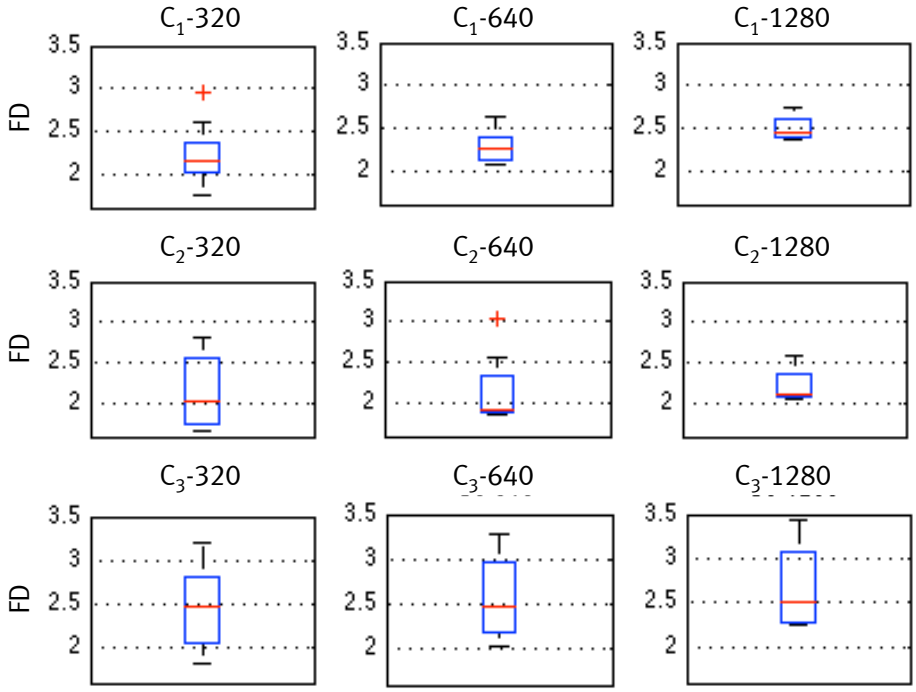

Figure 4.6. Boxplot representation of the fractal dimension data obtained by the Katz-Castiglioni algorithm, for each case (C_1 upper, C_2 middle and C_3 lower row) and window length (320 left, 640 middle and 1,280 right column).

Table 4.2. Medians of the fractal dimensions obtained for each sliding window length (320, 640 and 1,280) in C_1, C_2, and C_3. Note how close are the medians for C_3.

Case	Sliding window length		
	320	**640**	**1,280**
C_1	2.1415	2.2624	2.4420
C_2	2.0449	1.9188	2.1043
C_3	2.4752	2.4618	2.5207

The Shannon and permutation entropy values were calculated on the same data as the fractal dimensions, i.e., the trajectories displayed in Figure 4.4, and they are shown in Table 4.3. There was no difference between the Shannon entropy calculated on the X or the Y values. It was noteworthy the large difference between the entropy of C_3 and those of C_1 and C_2, which only differed slightly from each other (Table 4.3). This seems to indicate that the Shannon entropy decreases with increasing perturbation of the fish: tagging having only a minimal and possibly non-significant effect, but the presence of the contaminant drastically decreasing the entropy of the system by an entire unit.

Table 4.3. Shannon and permutation entropy values calculated for the X and Y coordinates for C_1, C_2, and C_3.

Case	Shannon Entropy values		Permutation Entropy values	
	X Coordinate	Y Coordinate	X Coordinate	Y Coordinate
C_1	6.3016	6.3016	3.0881	3.0950
C2	6.2861	6.2861	3.1049	3.1250
C3	5.3628	5.3628	3.0413	3.0618

Finally, the permutation entropy values calculated for the three experimental cases are also shown in Table 4.3. The results differ very slightly for X and Y signals and, in contrast to the results obtained using Shannon entropy, the three analysed cases presented very similar permutation entropy values. In this case as well, the permutation entropy values for C_3 were smaller than for C_1 and C_2.

Following the results of the tested methodologies, two of them, namely those based on the analysis of the Katz-Castiglioni fractal dimension and the Shannon entropy of the trajectories, have been shown to be potentially useful tools for non-invasive identification and quantification of changes of fish responses due to, primarily, a highly relevant environmental contaminant and secondarily, and probably in a much lower degree, to the variation in the number of fish in the system.

4.4. Conclusions

In conclusion, the present chapter describes a method for image acquisition, processing and nonlinear trajectory analysis suitable to identify variations in the response of a fish group to an event. The methodology here proposed shows a clear potential to aid implementing intelligent aquaculture systems. We believe that it will be possible to embed this methodology in an on-line/real time architecture to monitor fish schools in a farm and in the wild, and that this kind of approach will find an application to identify contaminated waters in environmental monitoring programs. In further studies the results reported here should, in author's opinion, be tested and validated with more contaminants, stressors and fish species, prior to be embedded in on-line monitoring systems using artificial intelligence methods.

Technically, the method presented here demands a relatively large computation capability, particularly for the image-processing step, which is of course susceptible of improvement. It must also be kept in mind, on one hand, that the analysis of the fish clusters trajectories does not depend on an image, they can also be obtained from echo-sounds or infrared images and, on the other hand, that the methodology is not exclusive for fish and that with some modification may be applicable to other species.

CHAPTER 5

VARIATION IN THE SHANNON ENTROPY OF THE TRAJECTORY OF A EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) SYSTEM IN RELATION TO THE NUMBER OF FISH

5.1. Introduction

As already addressed in Chapter 1, global fish production has been steadily growing during the last 50 years with most of the recent growth coming from aquaculture. Aquaculture production will need to continue increasing its output in order to contribute to the supply of high value nutrients to an exponentially increasing human population (Anon, 2011; FAO, 2014; Kalogerakis et al., 2015). This situation forces the aquaculture industry to face several challenges, including the need for an increased supply of feeds, access to clean water and enough energy, and competition from other activities (i.e. fisheries, tourism, industry) (Bostock et al., 2010; European Aquaculture Technology and Innovation Platform-Eatip, 2012).

Current aquaculture systems include freshwater ponds and tanks, freshwater cages, coastal ponds and tanks, coastal cage farms and systems for marine molluscs and aquatic plants (Bostock et al., 2010). New developments and trends are also focusing on closed systems, for example Recirculating Aquaculture Systems (German Advisory Council on Global Change - WBGU, 2013; NACA/FAO, 2001). However, state of the art research activities indicate that large scale aquaculture production systems, such as offshore exploitations and/or implementation of aquaculture activities within offshore multipurpose structures, may be a solution to some of the challenges (European Aquaculture Technology and Innovation Platform-Eatip, 2012). One important part of these platforms is the design of intelligent structures, i.e. structures able to register and respond to a changing external environment (such as loads and shape change) as well as to a changing internal environment (such as damage or failure). In contrast to the increasing amount of works devoted to the study of the physical design and intelligent design of the aquaculture platforms themselves, there are few works devoted to the automatic monitoring of the organisms being farmed, i.e. fish, which should be a part of the total intelligent system. To monitor the fish is important for several reasons: one is to know how many fish there really are and detect - optimally avoid - escapes, another is the early detection of abnormalities in their behaviour that may be an indication of disease, parasites or the presence of contaminants and, related to it, the third reason is to monitor the welfare of the fish under production.

At this point one of the variables to take into account is the number of fish to monitor. The studies published used different number of individuals some use only one fish (Brodin et al., 2013; Magalhães et al., 2007), others a few [10 fish, (Ladu et al., 2015) and 19 to 26 fish (Eguiraun et al., 2015b)] and others many (81 fish) (Eguiraun et al., 2014). There is usually no explanation regarding the criteria used to select the number of individuals, except in the cases where one wishes to study, or to avoid, the effect of stress by crowding. Even in the last case, the numbers are selected based on the empirical knowledge generated for a few species. However, collective animal behaviour differs from the behaviour of the individuals alone, and the behaviour of each individual within its group (be it a human in a crowd, a bird in a flock, an insect in a swarm or a fish in a shoal) is a combination of genetic and environmental factors [see the review by Sumpter (2006) and references therein]. Thus, analysing the

individual responses, one should expect a larger variation that analysing the response of the group, and yet, when one analyses a group composed of *many individuals* the response of the group resembles the average obtained from studies with individuals [see the review by Sumpter (2006) and references therein]. The key issue here is to identify the critical number, *n*, corresponding to *many individuals*. When using only one fish, it will very likely not represent the entire system and therefore one should set N experiments with only *n=1* fish (using a different individual for each experiment) so that the average of all the responses from all experiments represents the response of the system. In this case, one must know the value of *N*, i.e. the *number of experiments* one must perform to obtain a value representative for the population.

In order to set up a Biological Warning System one needs to identify the changes, in the individual or in the group, that occur in response to the introduction of perturbations (i.e. contaminants or stressors) into the system, but also the number of individuals used in the experimental phase must be reduced to a minimum for ethical and economical reasons. In real-life conditions in offshore cages it is very difficult to use the entire production (there may be several hundred thousand fish in a cage) as Biological Warning System, so the farmer may need to set up a smaller unit to monitor and serve as the Biological Warning System. In this case it is also desirable to use the smaller possible number of fish. On the other hand, the entire production unit might be used as a Biological Warning System when dealing with closed aquaculture systems, such as the increasingly popular recirculating aquaculture systems, or RAS.

In previous studies (Eguiraun et al., 2014) and confirming other authors' works (Bae and Park, 2014; Forlim and Pinto, 2014; Kadota et al., 2011; Liu et al., 2011a; Quach et al., 2013; Spasic et al., 2011) the Shannon entropy of the system was identified as a variable that changes with the introduction of perturbations into the system and has therefore the potential to serve for its monitoring.

For species that shoal, such as the European seabass (Pickett and Pawson, 1994) one could expect that, in addition to the variability in each individual's responses, individual isolation itself may have an effect and therefore one individual may not be the optimal number to select. However, the optimal number itself remains unknown. Probably, there may be a critical size/number of individuals after which further increase in the *number of individuals* does not add more information on the system, as it has been shown in ants by Sumpter (2006) and that would represent the *n* number of *many individuals* mentioned above whose study might be used to obtain a response representative for the real life system.

Accordingly, the present case study was designed to understand how the fish biomass variation affects the system dynamics in order to answer the following questions: i) Does the Shannon entropy of a fish system vary according to the number of fish? ii) If it does vary, how is this relationship? and iii) Is it possible to identify the number of *many individuals* to be used in future works to simulate the behaviour of a population in experimental settings or in a Biological Warning System?

Given the shoaling nature of the European seabass, it was expected to find a critical difference in the response of the system when using only one or very few fish. Thus, in order to characterize the biomass variation from 1 to 50 individuals two different experiments where performed: i) a decreasing biomass experiment starting with 50 fish and decreasing the biomass to 25, 13 and finally 1 fish and ii) an increasing biomass experiment, studying the system with initially 1 fish, and then adding 1 new fish per day during 5 days, i.e., ending with 5 fish in the tank.

5.2. Materials and methods

5.2.1. Animals and acclimation conditions

The fish were European seabass (*Dicentrarchus labrax*) generously provided by Grupo Tinamenor (Cantabria, Spain). In the Research Center for Experimental Marine Biology and Biotechnology - Plentzia Marine Station of University of the Basque Country UPV/EHU, they were acclimatized for 3 months in two flow through 1,800 L epoxy-coated fibreglass tanks containing aerated, naturally sand filtered seawater pumped from the Cantabric Sea in the North of the Iberian Peninsula (43°24'49.5"N 2°57'06.5"W). During this period, the seawater conditions oscillated according to the environmental variation but they were always within the values for optimal growth for the species. The fish were fed INICIO Plus feed from BioMar (56% crude protein, 18% crude fat) following the manufacturer specifications for fish size, biomass and water temperature. According to its size fish were considered sexually immature (Fishbase.org, 2015).

The length and weight of the fish for the decreasing biomass experiment are shown in Table 5.1 and the total biomass of the increasing biomass experiment is shown in Table 5.2.

Table 5.1. Experiment A: Decreasing number of individuals. Biomass in the beginning of the experiment. Tanks 1 and 2 were filled with 50 fish each.

n = 50 fish	TANK 1		TANK 2	
	Size [mm]	Weight [g]	Size [mm]	Weight [g]
Avg	159.5	36.02	158.1	35.28
Max	200.0	60.00	197.0	64.00
Min	135.0	18.00	130.0	17.00
Median	154.5	33.50	156.0	33.00
Total biomass [gr]		1,801		1,764

Table 5.2. Experiment B: Increasing number of individuals. Daily biomass variation in each tank.

Day number	TANK 1		TANK 2	
	Fish name	Total weight [g]	Fish names	Total weight [g]
1	*a*	77	*b*	78
2	*c*	51	*a,b*	155
3	*d*	53	*a,b,c*	206
4	*e*	58	*a,b,c,d*	259
5	*f*	53	*a,b,c,d,e*	312

5.2.2. Experimental conditions

All the parameters were monitored daily. The salinity, measured using a multiparametric meter HANNA HI98192, was 33 gr/L. O_2 saturation was measured using the JBL O_2 kit and it always was >80%. Water temperature, pH and ammonium were monitored using a thermometer (±0.5°C), a CRISOM pH-meter Basic 20+ and Sera NH_4-NH_3 ammonium kit respectively. Water flow (fixed at 0.54 m^3/h) and additional air supply diffused by a stone were kept constant, interrupted only during the time necessary for recording the fish in order to avoid artefacts in the images. The experiments were performed in the period November-December during which only small variations were detected in the seawater temperature and pH following the usual seasonal changes (Table 5.3).

Table 5.3. Water/environmental conditions. Minimum and maximum values in relevant seawater parameters during the experimental period (Nov-Dec).

	Min	Max
Temperature [°C]	16.9	18.5
pH	7.76	7.93
Ammonium [mg/L]	0.0	0.0
Water flow [m^3/h]	0.54	0.54
Salinity [g/L]	33	33
O_2 Saturation	>80%	>80%

Two identical fiberglass tanks were used (100 cm x 100 cm x 90 cm) under direct white artificial light (2 x 58 W and 5,200 lm), avoiding the formation of shadows into the tanks and using the same light conditions in both. The tanks, equipped with a flow through system, were filled up to 9 cm from the upper border with 810 L of naturally sand filtered seawater. One camera was placed in each tank and exactly in the same position in both tanks, obtaining in both situations the same visual angle. The photoperiod was fixed at 12h/12h dark/light. The seawater was naturally sand filtered in its way from the sea to the aquarium. Data acquisition was done by video camera recording using the same experimental setup described by Eguiraun et al. (2014).

5.2.3. Experimental setup

Two experiments were run using the previously mentioned tanks and conditions. In the first experiment, Experiment A, which was run in duplicate, the number of fish was reduced from 50 to 1 fish in 4 steps (Figure 5.1). In the second experiment, Experiment B, the number of fish (but not the fish itself) was kept constant in tank 1, and it increased from 1 to 5 fish in 5 steps in the second tank (Figure 5.2).

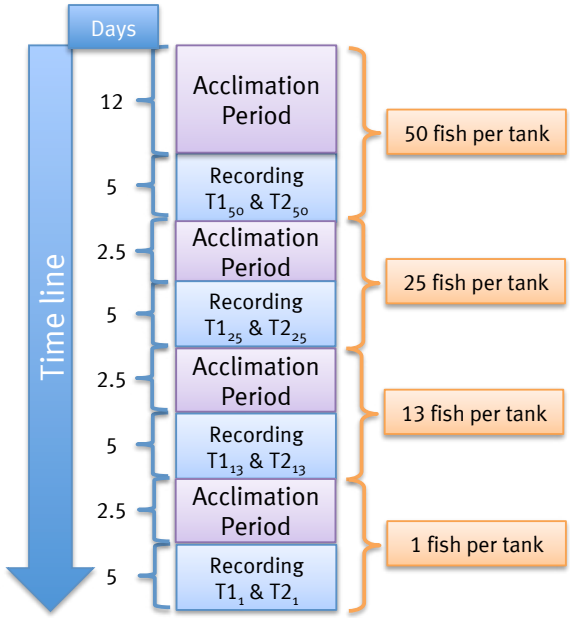

Figure 5.1. Description of Experiment A. Experimental setup of the decreasing density experiment. The number of fish was halved in each step except the last one, when it was reduced from 5 to one individual. T1 and T2 indicate tanks 1 and 2 respectively and the sub-index the number of fish. The number of days the activities lasted is shown under "Days" on the left.

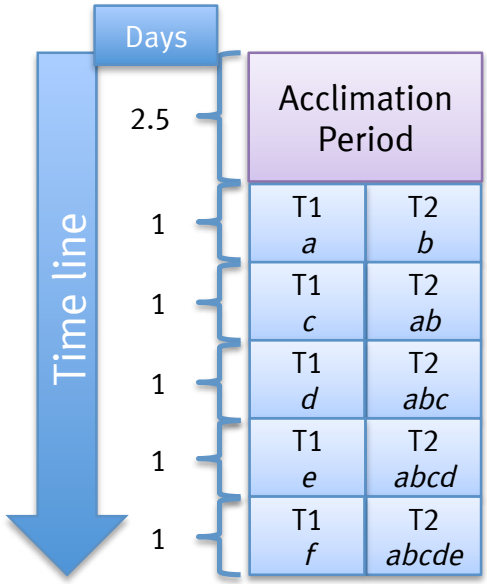

Figure 5.2. Description of Experiment B. Experimental setup of the increasing density experiment. The number of fish was kept constant, only one fish, in tank 1. Each day the fish that had been for one day in tank 1 was transferred to tank 2. Thus, the number of fish in tank 2 increased by one individual every day. T1 and T2 indicate tank 1 and 2 respectively. The number of days the activity lasted is shown under "Days" on the left; each letter within the tanks; *a, b, c, d, e* and *f,* refers to an individual fish.

5.2.3.1. Experiment A - Decreasing the density

Each of the two replicate groups consisted of 50 fish with a biomass as similar to each other as possible (Table 5.1). The fish were acclimated for 12 days to the new conditions and they were monitored and recorded during the next 5 days following the procedure described below. After that, both groups where reduced to 25 fish, trying to maintain a similar biomass in both groups. The remaining 25 fish per group were acclimated for another 2.5 days and subsequently monitored and recorded for 5 days. Past those 5 days both groups were reduced to 13 fish per group, acclimated for 2.5 days and recorded for 5 days. Finally, the groups were reduced to only one fish. Again, after 2.5 days of acclimation, they were recorded for the final 5 days of the experiment (Figure 5.1).

5.2.3.2. Experiment B - Increasing density

The experimental schedule is resumed in Figure 5.2. In this particular case, and during the five days the experiment lasted, tank 1 had only 1 fish and every day the fish that had been one day in tank 1 was transferred to tank 2 and a new fish was placed in tank 1. The new fish introduced every day in the experimental tank was taken from the acclimation tank not used for the experiments but maintained under the same conditions and in the same room. For a better understanding of the procedure, each fish has been named with a letter from *a* to *f* in Figure 5.2 and Table 5.2. The biomass variation and weight of the fish are summarized in Table 5.2. The fish were recorded the day after the change of tank.

5.2.4. Data acquisition

Data acquisition was performed by video camera recording using the same experimental setup described in Chapter 4. In short, recording was performed using a GoProHero3 camera with underwater housing inside each tank. Raw data were recorded in 1440p high definition format, 24 frames per second (fps) and 4:3 video size and it was stored in SanDisk 32Gb UltraMicroSDHCTM (Class 10) secure cards.

As already mentioned, the water flow and air intake were halted during the recording period to avoid bubbles and disturbances in the images. The recording took place during 1 hour per day and approximately in the middle of that period a stochastic event consisting of a hit in the tank was introduced. The images to be processed consisted of three measures of the basal state, of 3.5 min each, and the 3.5 min containing the hit in the tank, i.e. the stochastic event, as described in Chapter 4 (Figure 5.3).

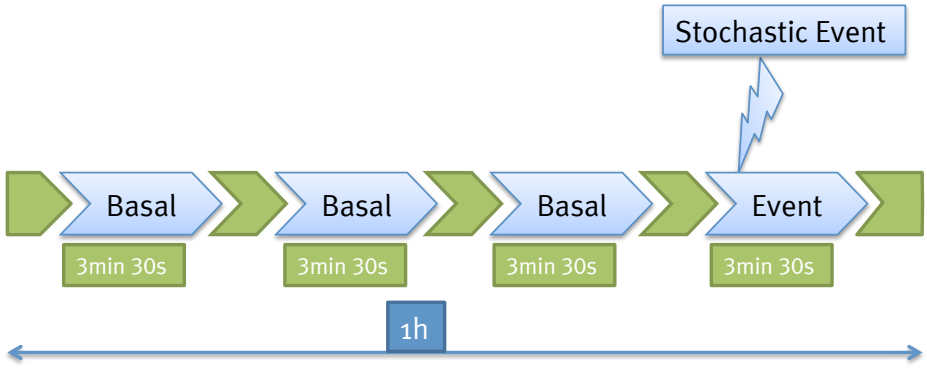

Figure 5.3. Recording procedure. Three basal and one event response measurements were processed from the total recorded period of 1 hour.

5.2.5. Image processing

It was performed as described in Chapter 4. Once the wanted videoclips (3 basal and 1 event per tank and per day) were located in the 1h recording, they were transformed into a 640 pixel x 480 pixel format image sequences per video clip at 24 fps using the iMovie commercial software and MPEG Streamclip free software. Subsequent image and feature extraction were carried out with Matlab running on a MacBookPro 2,6 GHz Intel Core i7 laptop with a SSD storage disk and 16 Gb of RAM.

5.2.6. Trajectory Estimation

The methodology used from image acquisition to fish group centroid trajectory estimation depicted in Figure 5.4, was based on that described in Chapter 4 with some modifications. Firstly the trajectory of the cluster's centroid was built computing the elements center's in every single frame, which leads to a very noisy signal unsuitable for the later non-linear signal analysis. Thus, the noise of the signal was reduced calculating the cluster's centroid applying the k-means algorithm to the number of elements in each frame using the centers of the elements in the first frame as input coordinates. Secondly, the trajectories in X and Y were analysed in the same format they were obtained although they have different scale dimensions. X trajectories have dimension from 0 to 640 and Y trajectories have dimension from 0 to 480 due to the pixel image size. The results indicated that analysing those raw trajectories leads to satisfactory results and differences were not found between the results obtained analysing the raw and the normalized trajectories. However, and with the purpose of building a more robust algorithm for future applications in mind, the X and Y trajectories presented in the current work were normalized using z-score technique.

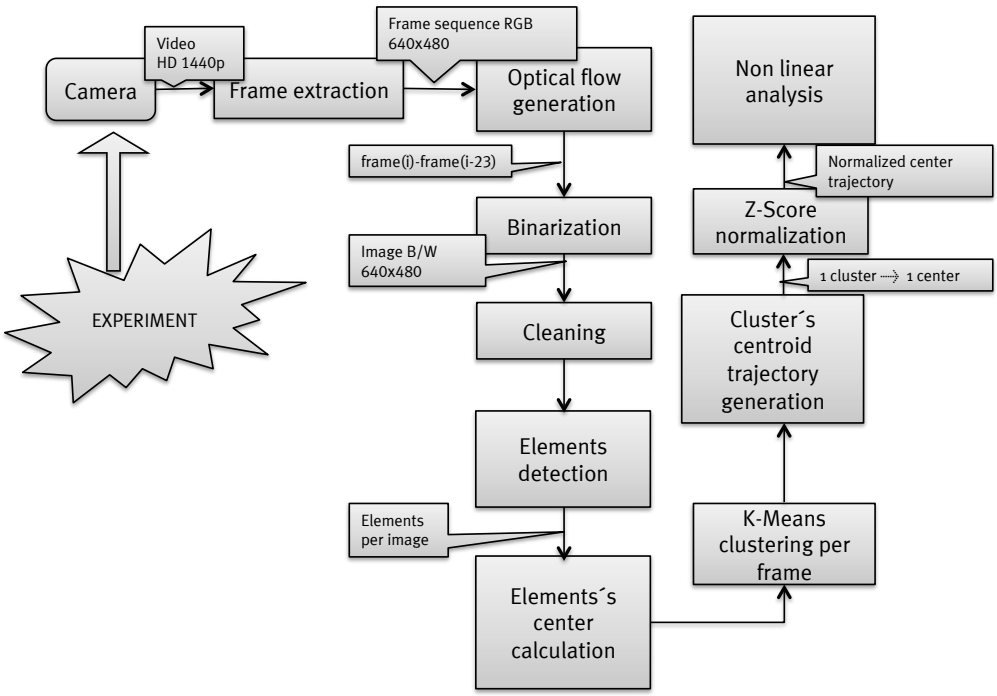

Figure 5.4. Data acquisition and processing workflow. Based on the one described in Chapter 4 and with the two variations described in the text.

5.2.7. Non Linear Trajectory Analysis

As described in Chapter 4, Shannon entropy was selected as the best parameter to analyse the trajectories due to its low computational load and robustness.

5.3. Results

5.3.1. Experiment A

Table 5.4 shows the daily evolution in the two experimental tanks of the Shannon entropy corresponding to the basal trajectories (blue points in Figure 5.5) and to the trajectories followed in response to the stochastic event (red points in Figure 5.5). As shown in Table 5.4, the response obtained in both tanks was very similar, and the Shannon entropy of the basal trajectories represented in the figures correspond to the average of six different measurements (three in each tank) while only one was obtained in response to the event. These results showed that the Shannon entropy of the system increased concomitantly, but not linearly, with the number of fish. In addition, while the Shannon entropy of the response and basal trajectories in tanks with 13 or more fish had similar values, the Shannon entropy of the system in response to the stochastic event was clearly higher than the Shannon entropy of the basal state with only one fish.

5.3.2. Experiment B

The average values of the Shannon entropy of the basal trajectory in the 1-fish system kept values close to, but slightly lower than 2, except for the last fish that displayed a value much lower than 1 (upper panel in Figure 5.6). It must be noted than in this case it was not possible to talk about trends, since each day there was a different fish in the tank. The Shannon entropy of both the basal and response trajectories increased with increasing number of fish between 1 and 4 individuals (lower panel in Figure 5.6). Further increase from 4 to 5 fish modified only slightly the values of the Shannon entropy of both trajectories.

The results obtained for the Shannon entropy as a function of the number of fish, for both the basal and response trajectories and from 1 to 50 fish (i.e. adding the results of Experiments A and B), fitted an exponential curve whose parameters are described in Table 5.5 and plotted in Figure 5.7 for the basal (top panel) and the event-response (bottom panel) trajectories.

Table 5.4. Daily evolution of the Shannon entropy in Experiment A in tanks 1 (T1) and 2 (T2). Shannon entropy of the basal (average of six different measurements: 2 tanks and 3 measurements per tank) and event responses are shown.

# fish	Tank #	Day 1		Day 2		Day 3		Day 4		Day 5	
		Basal	Event	Basal	Event	Basal	Event	Basal	Event	Basal	Event
50	T1	4.92±0.14	5.16	4.98±0.15	5.23	4.73±0.11	5.00	4.79±0.04	4.77	5.09±0.28	4.82
	T2	4.62±0.07	4.62	4.66±0.09	5.68	4.60±0.04	4.89	4.63±0.08	4.84	4.68±0.10	4.81
25	T1	4.71±0.01	4.85	4.47±0.10	4.53	4.50±0.14	4.41	4.30±0.05	4.75	4.46±0.11	5.46
	T2	4.76±0.04	4.98	4.78±0.22	4.76	4.67±0.11	4.73	4.67±0.10	4.58	4.69±0.36	5.41
13	T1	4.11±0.23	4.27	4.05±0.11	3.88	4.75±0.45	4.43	4.20±0.34	4.05	4.34±0.55	4.40
	T2	3.97±0.17	4.47	3.99±0.20	4.40	4.21±0.27	4.16	3.99±0.08	4.02	3.97±0.08	4.06
1	T1	0.59±0.39	2.79	1.84±0.63	2.63	0.97±0.77	2.26	1.34±0.34	2.15	0.87±0.03	1.49
	T2	0.52±0.23	2.79	0.73±0.19	1.63	0.38±0.13	2.07	1.40±0.47	1.50	2.01±0.49	2.31

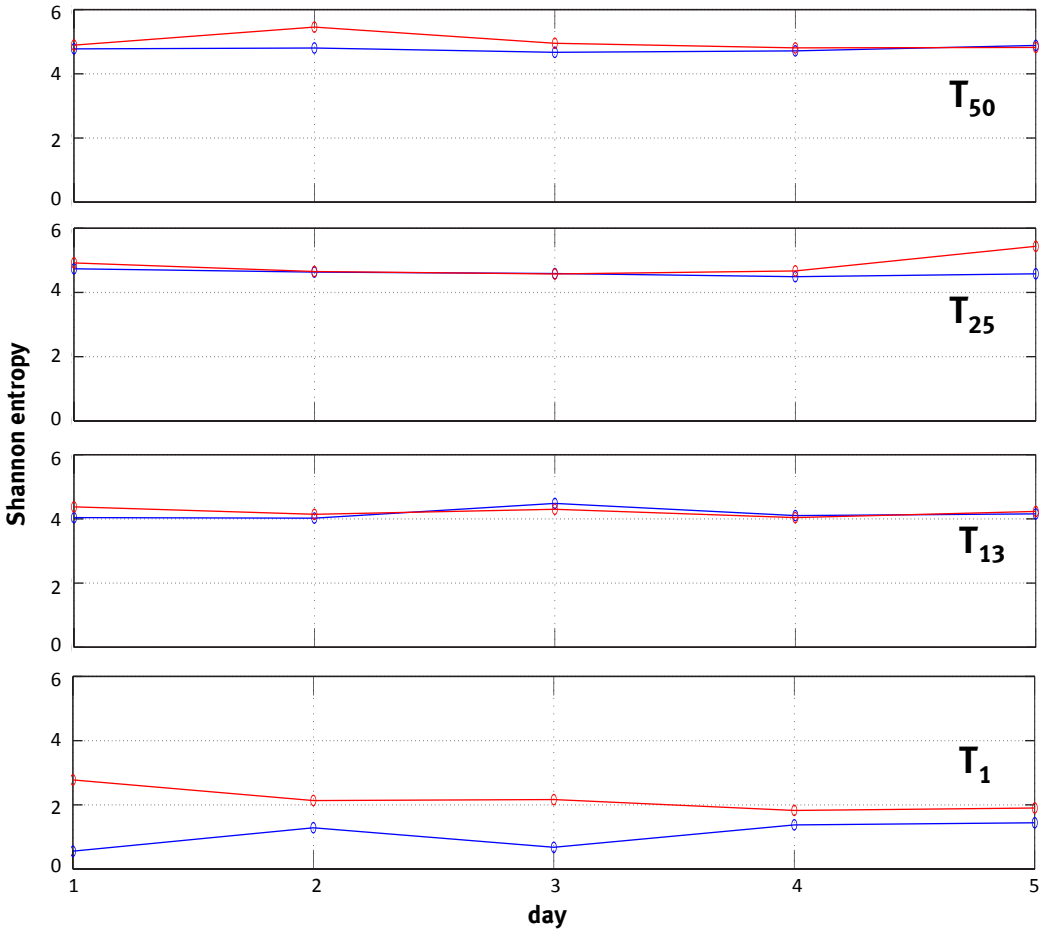

Figure 5.5. Daily evolution of the Shannon entropy in Experiment A. The blue points correspond to the Shannon entropy values for the basal states (average of six different measurements: 2 tanks and 3 measurements per tank) and the red to that of the response to the event. The lines are plotted to join the points.

Table 5.5. Curve fitting parameters and goodness of the fit of the Shannon entropy vs. the number of fish. The Shannon entropy values *(y)* of the basal state and of the event response were fitted as a function of the number of fish (*x*). *u*, *v*, and *w* are the coefficients of the curve. The goodness of the fit was estimated by the sum of squares due to error (SSE), R-square, adjusted R-Square and root mean squared error (RMSE).

$y=u\cdot x^v+w$		Basal	Event
Coefficients	*u*	-4.102	-11.09
	v	-0.4957	-0.07018
	w	5.382	13.5
Goodness of the fit	SSE	0.04477	0.2228
	R-Square	0.9953	0.9619
	Adjusted R-square	0.9934	0.9467
	RMSE	0.09463	0.2111

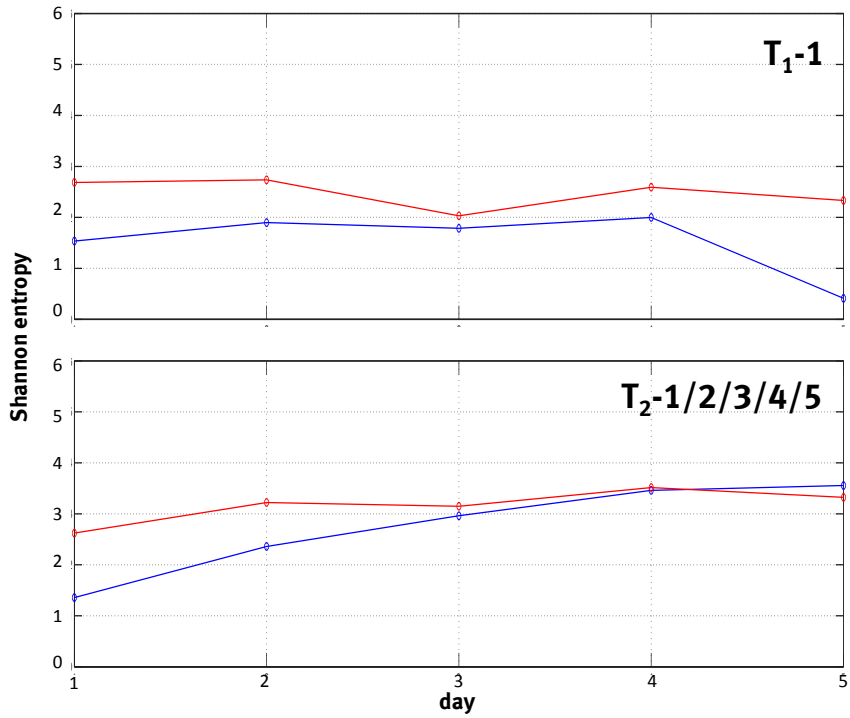

Figure 5.6. Daily evolution of the Shannon entropy in Experiment B. T_1 (upper panel) contained only one fish, but a different fish every day, during the 5 experimental days. The number of fish in T_2 increased by one individual daily (lower panel). The number of fish is indicated on top of the panels. The blue points correspond to the Shannon entropy values for the basal states and the red to the response to the event. The lines were plotted to join the points.

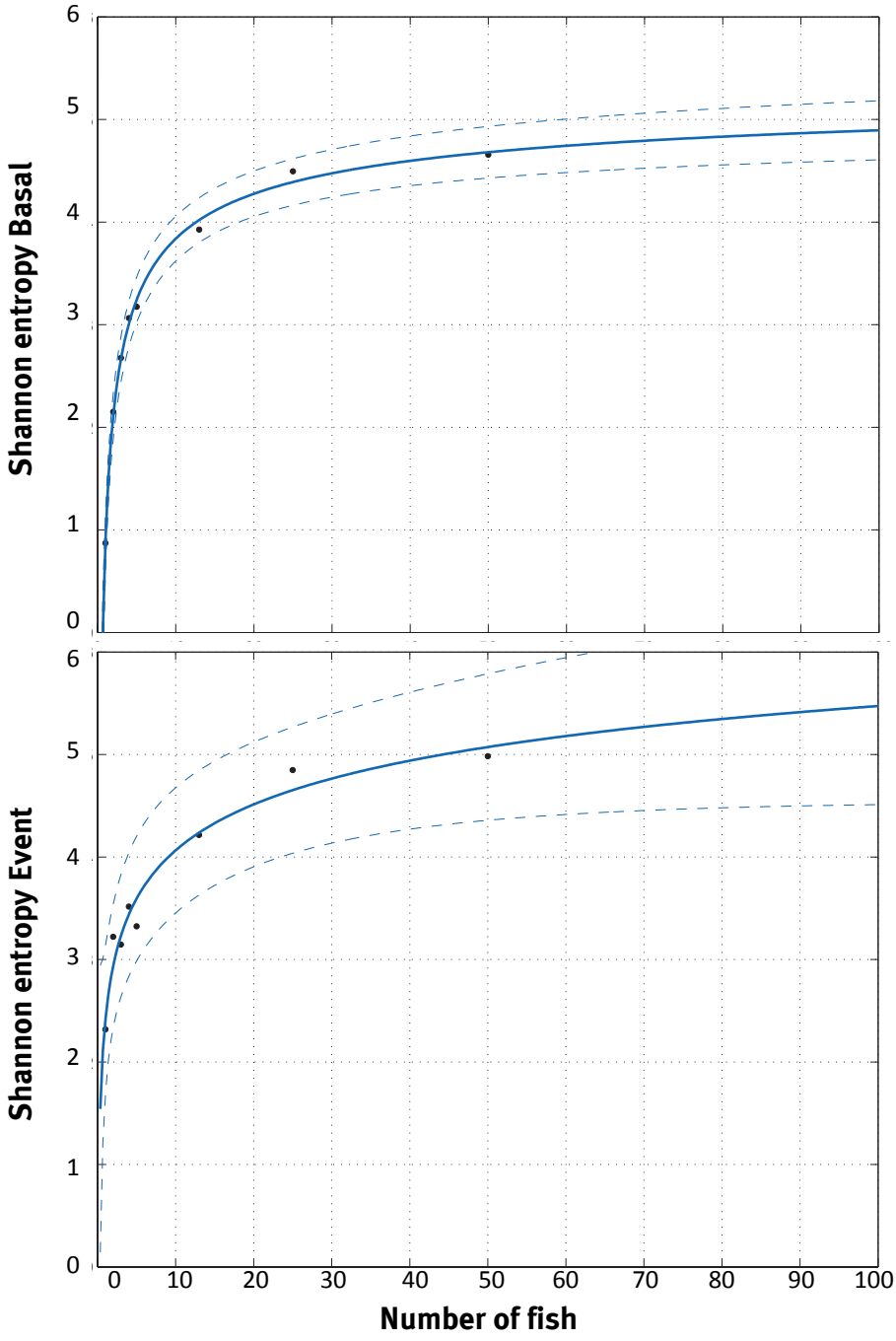

Figure 5.7. Curve fitting of the Shannon entropy as a function of the number of fish. The basal state (top) and the response to the event (bottom) are shown together with the 95% confidence bounds.

5.4. Discussion

The expected increase in aquaculture activities and production in fish farming, particularly in Recirculating Aquaculture Systems, will demand the development and application of on-line, non-invasive and cost-effective monitoring systems (Kalogerakis et al., 2015) for which, the present Thesis proposes the implementation of a Biological Warning System where the fish under production constitute the system to monitor. The aim of the present Chapter was to obtain an essential piece of information: to elucidate whether the number of fish affected the Shannon entropy of the system in a known shoaling fish species (European seabass), and, if so, what type of relationship these two variables kept. It must be noted that we did not aim at mapping behavioural characteristics such as time swimming or resting, aggressive behaviour or the shoaling itself, which would require a different methodological approach.

The present work showed that: i) the Shannon entropy of the European seabass system is highly dependent on the number of individuals for a few fish (from 1 to 5) becoming more independent from the number as it increases and that ii) this dependence nicely fits an exponential curve.

The concepts shown here may apply not only to European seabass, but also to other similarly shoaling species. Although the behaviour and response of the system will likely be species-specific (Boerrigter, 2015), this approach might be applied with few modifications to monitoring salmon, charr, cod, trout and similar shoaling species, although probably not to species like eels or flatfish that have different swimming patterns and, probably, also different response dynamics. One work with sticklebacks by Wark et al. (2011) is particularly interesting because it showed that the application of Shannon entropy to signal processing analysis uncovered information that classical analysis tools did not and, in addition, demonstrated that the behaviour of their fish biological system had a clear genetic component. They calculated the entropy of the distribution of the individuals within the shoal while we calculated the entropy of the trajectory of the centroid of all the fish; which does not tell us whether the fish shoal or not (information that this work is not targeting). In spite of this basic difference it is very interesting to note that both methods measured higher entropy values with (i) increasingly random distribution of fish within the shoal and (ii) increasingly random trajectory of the centroid. Taken together Wark et al. (2011) and the present work, it seems that the implementation of a Biological Warning System within a Fish Welfare Assurance System and/or a Hazard Analysis and Critical Control Points plan in aquaculture production has a real potential of being successful for different species, and that the monitoring system must use the same species and stock than the individuals to be evaluated, since the environment from which the fish originated may dictate their behaviour not only in the wild but also in farming and laboratory settings.

For European seabass monitoring it seems that a number between 5 and 13 individuals may be the lowest suitable number to achieve meaningful results —for example to perform experiments or to set up a Biological Warning System monitoring unit— and correspond to the number *n* of *many individuals* mentioned in the introduction. However, in order to develop a model applicable to a larger population

(in particular if intended to be used in real-life Recirculating Aquaculture Systems monitoring of entire production units), there is a need to identify also the number corresponding to *too many individuals*, i.e. the number of individuals that would make the system collapse. For example, overcrowding may limit the space where each animal can move, which will in turn make the movement of the centroid of the shoal appear increasingly stagnatic, regardless of whether the individuals themselves move or not. In this latter case the Shannon entropy value of the system may revert to lower values, or even become zero for a completely static centroid. Thus, it is necessary to identify the values of both *many individuals* and *too many individuals* within which the results are valid. For the applications targeted by this work, i.e., implementation of a Biological Warning System in aquaculture and for experiments that require fish, we consider that 5-13 fish may be adequate using our tanks and conditions.

CHAPTER 6

APPLICATION OF THE TOOL TO AN EXPERIMENTAL CASE: VARIATION OF THE SHANNON ENTROPY IN A EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) SYSTEM EXPOSED TO SODIUM SELENITE (Na_2SeO_3) AND/OR METHYLMERCURY CHLORIDE (CH_3ClHg)

6.1. Introduction

As already mentioned, one of the challenges to the aquaculture industry is the lack of non-invasive, fast, easy, inexpensive methodologies to estimate the welfare of the fish and/or the detection of diseased and contaminated fish groups (Eguiraun et al., 2015a). The entrance points for undesirable agents which affect fish welfare by infecting or contaminating them are usually the prey/feed, veterinary treatments (for farmed fish) and the environment for both farmed and wild fish (Dahle et al., 2010). Monitoring of the critical points both for wholesomeness in a Hazard Analysis and Critical Control Points plan (Huss et al., 2004) and for welfare in a Fish Welfare Assurance System (van de Vis et al., 2012) demands the identification of the points themselves, a monitoring method adjusted to the parameter one wishes to measure and the establishment of an Optimal Working Point (OWP), or range, i.e. the value or values within the critical limits that indicate that the system is operating under optimal conditions. When the value registered during the monitoring is outside the Optimal Working Point, i.e. higher or lower than the maximum and minimum critical limits respectively, and alarm goes off and corrective actions must be taken until the deviation is corrected and the Optimal Working Point values are restored.

Current practices in aquaculture do not usually include the automatic monitoring of the behaviour and/or responses of the fish. Previously in this Thesis, the application of Biological Warning Systems approach to monitor fish production in aquaculture has been proposed in Chapter 1 (Eguiraun et al., 2015a), a tool for this purpose has been developed in Chapter 4 (Eguiraun et al., 2014) and the response of the system as a function of the number of fish has been characterized in Chapter 5.

The purpose of this Chapter was to test the tool in an experimental setting where the fish had been exposed to 2 different substances: sodium selenite (Na_2SeO_3) and methylmercury chloride (CH_3ClHg, abbreviated as MeHg). For that test, we used the experimental set up established for a study parallel to this PhD Thesis on the biochemical and histological effects of the pre-administration of Na_2SeO_3 on MeHg toxicity on European seabass. This offered an excellent opportunity to test the tool on a real-life experiment with negative and positive aspects. A negative aspect was that the experiment had not been designed to optimize the monitoring conditions; it had been designed and optimized (among other things, by mixing Na_2SeO_3 pre-treated and not pre-treated fish in the same tank and by adding the contaminants to the water) for a biological study. However it also presented some exciting challenges and positive aspects as well, namely that these were conditions one could encounter in real life such as non-homogeneous populations of fish (some with high and other with low levels of contaminants) and high water turbidity.

The main reason to select these two compounds, as already indicated in Chapter 1, was that MeHg is a known environmental contaminant of increasing concern for the fish industry and consumers alike and considered a risk factor in fish consumption. MeHg is a neurotoxic substance that reaches high levels of accumulation in the central neural system (Berntssen et al., 2003). Chronic exposure to it induces (among many others) symptoms similar to those observed in amyotrophic lateral sclerosis, such as

the early onset of hind limb weakness in humans (Berntssen et al., 2003; Johnson and Atchison, 2009). Selenium compounds on the other hand, particularly those present in fish, display very high affinity for MeHg, and have been proposed as capable of neutralizing its toxic effect in addition to showing a high antioxidant effect (Ralston 2008; Ralston et al. 2008; Raymond et al. 2012; Yamashita and Yamashita 2010; M. Yamashita et al. 2013; Y. Yamashita et al. 2013).

Knowing, as described above, that MeHg affects the neural system in general, and the active swimming in salmon (Berntssen et al., 2003) we hypothesized that exposure to MeHg would affect the Entropy of the trajectory of the fish system and change its value, taking it outside what could be considered the optimal working range and that removal of the contaminant would bring it back to the optimal working range.

Since previous knowledge also indicated that selenium might counteract the effects of MeHg, the administration of these two substances would theoretically keep the fish system within its optimal working point. However, there is a very narrow range between the optimal daily ingestion of selenium and a toxic dose which is dependent on the selenium compound (Nuttall, 2006). Na_2SeO_3 was selected for this work because it is a common form of selenium used as a supplement in foods and feed, used in previous experimental works (Branco et al., 2012) although its use and optimal dose is still subject to some controversy (Nuttall, 2006).

The experiment had been designed in three phases: during Phase A some fish were exposed to Na_2SeO_3; in Phase B they were exposed to MeHg and in Phase C the MeHg was removed, i.e. Phase C corresponds to the recovery period from MeHg toxicity.

6.2. Phase A - Na_2SO_3 exposure

6.2.1. Biological material and water/environmental conditions

European seabass (*Dicentrarchus labrax*, 4±2 g, 8±1 cm) generously provided by Grupo Tinamenor (Cantabria, Spain) had been transported to our lab in their own seawater with constant aeration and had been acclimated for 1 week in 1,800 L epoxy-coated fibreglass tanks containing aerated, circulating seawater at 13 ºC. During all the experimental period the fish were subjected to a 12 h/12 h dark/light photoperiod and they were fed once a day INICIO Plus feed from BioMar (56% crude protein, 18% crude fat) according to the manufacturer's specifications for fish size, biomass and water temperature.

The salinity was measured prior to the beginning of the experiment by a multiparametric meter HANNA HI98192, and it was 33 gr/L. The values of ammonia, nitrate, dissolved O_2, temperature and pH were kept within non-stressful values and their variation was insignificant during the experiment. The water used was pumped directly from the sea and it was sand filtered and treated with ion exchange resins in order to eliminate any possible contaminants. Additional O_2 supply (bubbled into the

tank) was introduced and interrupted only during the time necessary for recording the fish in order to avoid artefacts in the images.

6.2.2. Experimental setup and exposure to Na_2SeO_3

Two experimental cases were analysed of 76 fish each: C_1, which was the control group and C_2, which was the group exposed to Na_2SeO_3 (Sigma-Aldrich product number S5251-10G). Each experimental case consisted of a tank (100 cm x 100 cm x 90 cm), filled up with 810 L of aerated seawater to a height of 80.5 cm. In both cases the fish were acclimatized for 1 day (day 0). The tanks were placed under direct artificial white light (2 x 58 W and 5200 lm) avoiding shadows as much as possible.

Fish in C_2 were exposed during the next 6 days to a dose of 10 µg of Na_2SeO_3 per L seawater according to Branco (Branco et al., 2012). In order to ensure that the concentration of Na_2SeO_3 remained constant during the exposure period, the water flux was halted in both tanks (treatment and control) and the water was renewed on days 3 and 4, to reduce nitrogen residuals and dirt. After each change of water, fresh Na_2SeO_3 (10 µg/L) was added to C_2. The fish remained in the tanks during the changes of water in order to minimize their stress. Both tanks were treated equally, except for the addition of Na_2SeO_3 that took place only in C_2. As mentioned, the O_2 intake was halted during each day's recording period, to avoid bubbles and disturbances in the images. No mortality was registered during this Phase A.

6.2.3. Image acquisition, processing, trajectory estimation and non linear trajectory analysis

Image acquisition, processing, trajectory estimation and non linear trajectory analysis was carried out as described in Chapter 5. The basal status and event responses were measured both in the control group C_1 and in the treated C_2 but the data of the basal status of C_2 were unfortunately lost due a hardware failure in the hard disk were they had been stored.

As already discussed in the limitations of the study in Chapter 2, the turbidity developed in both tanks during the 48 h that the water was not being circulating was a challenge to the image processing platform designed. Also, as it has been previously mentioned, the images were processed using the same computational parameters regardless of the degree of turbidity in the water.

6.2.4. Results and Discussion

The day 0 of the experiment was considered an acclimation day and used as a control for the beginning of the experiment, thus data from this day were not used for the calculations. Table 6.1 shows the mean and standard deviation of the Shannon

entropy values for C_1 and C_2 from day 1 to day 6. The water of the tanks was changed in days 3 and 4 (denoted with *) and we expect the fish to have suffered some kind of stress due to this fact.

The results obtained in the control group, C_1, for both the basal and event responses confirm the results obtained in Chapter 5. On one hand, the Shannon entropy of the basal state is lower than that of the event, which can be explained because a system in a resting state disperses less energy than when it is excited, i.e., when the event is introduced. On the other hand, no significant differences were detected between the Shannon entropy values corresponding to the events of C_1 and C_2 and those calculated from the results obtained in Chapter 5 for the number of fish used here, i.e. 76, as shown in Figure 6.1.

These two facts suggest that the basal state may be more difficult to characterize than that of the event, perhaps due to arbitrary behaviour and different circadian cycles of the fish. However when an event is introduced, as it is a response to a sudden perturbation, the system responds in a more coherent manner, making the value of the Shannon entropy obtained in this case more reliable.

The Shannon entropy values of C_1 and C_2 in response to the event show that both systems have a similar behaviour in terms of energy exchange, i.e., that both systems disperse similar amounts of energy. From these results it can be concluded that the exposure to Na_2SeO_3 does not affect the energy balance of the fish system, thus Na_2SeO_3 exposure does not affect fish in a quantifiable manner using the proposed methodology. These results would agree with the expected effect of Na_2SeO_3: it should protect against MeHg toxicity, but not display any additional effect. In other words, exposure to 10 µg of Na_2SeO_3 per L water in the tank does not take the system outside its optimal working point, as far as its Shannon entropy can measure, and the farmer would not need to take any corrective action.

Table 6.1. Phase A-Exposure to Na_2SeO_3. The table shows the Shannon entropy values per day and experimental case. The data correspond to three basal state measurements and the averaged measure for the control case, C_1; and event responses for C_1 (control) and C_2 (exposed case). The water of the tanks was changed on the days labelled with an asterisk (*).

Day	C_1					C_2
	Basal	Basal	Basal	Average Basal	Event	Event
0	5.3707	5.3300	5.4184	5.3697±0.0492	5.6393	5.0132
1	4.6969	4.4265	4.6291	4.5842±0.1407	4.7242	5.1670
2	4.3904	4.0520	4.5787	4.3403±0.2669	5.0367	4.7467
3*	4.9046	4.7100	4.5365	4.7170±0.1842	6.2145	4.7868
4*	4.8874	4.8016	4.7104	4.7998±0.0885	6.0924	5.8601
5	4.6227	4.6259	4.5987	4.6158±0.0149	5.1643	5.2968
6	4.1976	4.0003	4.0387	4.0789±0.1046	4.3488	4.7703
Average				4.5227±0.0905	5.2635±0.7457	5.1046±0.4365

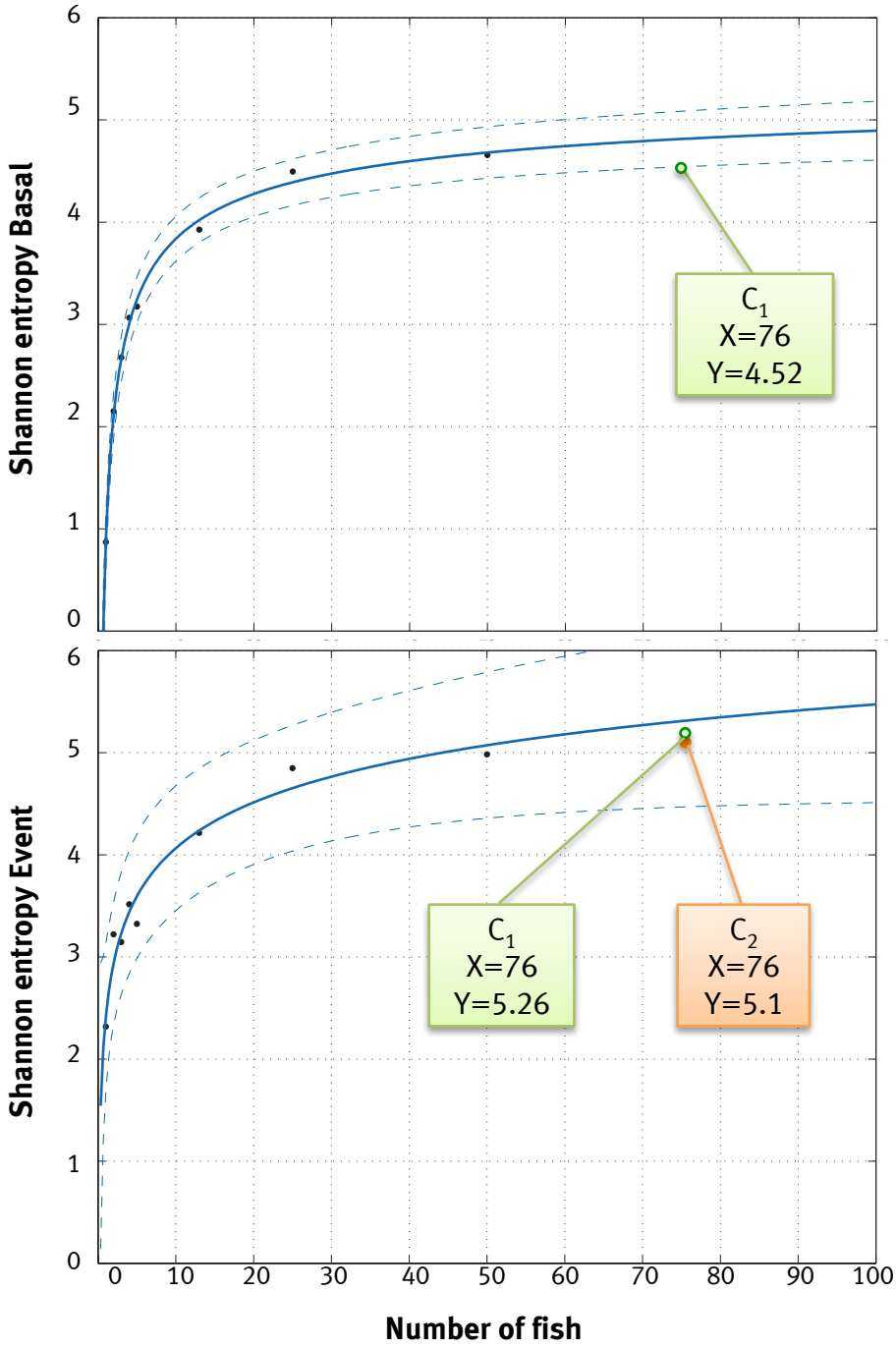

Figure 6.1. Shannon entropy values for the basal state (upper panel) and event responses (lower panel) for cases with different number of fish extracted from Chapter 5. The inserts indicate the value of the Shannon entropy for the event response of the control case C_1 (green) and of the Na_2SeO_3 treated case C_2 (orange) from the Phase A.

6.3. Phase B - Exposure to MeHg

6.3.1. Biological material, water/environmental conditions, experimental setup and exposure to MeHg

As already mentioned, this experiment was designed to study the (presumably protective) effect of the pre-exposure to Na_2SeO_3 on MeHg toxicity as measured by variations in some biochemical and histological parameters. At the end of Phase A, 10 fish from each tank were sacrificed for biochemical analyses, leaving 66 European seabass (*Dicentrarchus labrax*) in each tank. These 66 fish were divided into two groups of 33 fish each: 33 fish remained in the tank and the other 33 were transferred to the other tank. Thus the two cases now were made up of 66 fish each, but they consisted of mixed populations of fish, were half (33 fish) had been treated with Na_2SeO_3 and the other half (33) had not. During this Phase B one of tanks was exposed during 14 days to MeHg contamination (4 µg MeHg per L water in the tank) and the other was not. Both cases were subjected to the same conditions regarding frequency of water changes (every second day), which induced an unavoidable development of turbidity due to the impossibility of having the seawater circulating with a reliable constant and continuous MeHg concentration. MeHg was purchased from Sigma-Aldrich (product number 33368).

In this Phase B, C_3 becomes the new control group and C_4 becomes the MeHg treated group, and at the beginning of the MeHg treatment each group contains 33 untreated fish and 33 that had been treated with Na_2SeO_3.

6.3.2. Results and discussion

During this Phase B, as it occurred in Phase A, the seawater in the tanks was not circulated because of the need to maintain the MeHg concentration and also to avoid the large volume of contaminated water that would have had to be treated prior to being safely discarded, which would have significantly increased the costs and risks of the experiment. Although the parameters used as diagnostic for acceptable seawater quality (ammonia, nitrate, dissolved O_2, temperature and pH) were monitored daily and found to be within an acceptable range, turbidity developed in the 48 hours the water was kept still, as in Phase A, and it can be assumed that these conditions must have induce some degree of stress in the fish.

At the end of the first week of this phase, 10 fish per tank were killed for histological purposes and the same occurred at the end of the second week. In addition, mortality occurred several times during the 2-week exposure period in both experimental groups. These two factors made the biomass decrease in both tanks.

Taken into account the variation in the number of fish and the poor quality of the images obtained during most of the experimental period, we consider that

the results obtained using the methodology developed in this PhD were not reliable. However, the biochemical analyses indicated that MeHg-treatment had had a clear effect by drastically diminishing the activity of a liver enzyme called thioredoxin reductase and that the pre-treatment with Na_2SeO_3 had exerted a protective effect on its activity, i.e., the Na_2SeO_3 and MeHg treated fish had higher values of activity for that enzyme than the fish subjected only to MeHg contamination (Vitalle, 2014). Thus although the tool could not be evaluated during this phase, Phase B fulfilled its function by confirming that both the Na_2SeO_3 and MeHg had displayed the expected effects on the fish.

6.4. Phase C - Recovery period

6.4.1. Biological material, water/environmental conditions, image acquisition, processing, trajectory estimation and non linear trajectory analysis

The European seabass (*Dicentrarchus labrax*), which had survived Phase B, were used for Phase C. At the end of the second week of MeHg treatment, 10 fish from each tank were sacrificed for histological and biochemical analyses. The remaining fish in each tank were left to recover for the next 11 days, during which the treatments were withdrawn and the water was kept under constant circulation. The two new experimental cases during Phase C were: C_5, the control group of this phase that had not been treated with MeHg and consisted of 26 fish; and C_6, the group that had been treated with MeHg and consisted of 19 fish. As in Phase A - and unlike in Phase B - no mortality was registered during Phase C.

During the 11 days that Phase C lasted, both tanks were monitored daily and the Shannon entropy of the fish centroid's trajectory was measured during the event response. Image acquisition, processing, trajectory estimation and non-linear trajectory analysis were performed as in Phase A and in Chapter 5. The basal images of the current phase were also lost due to the same hardware failure that affected Phase A.

6.4.2. Results and discussion

During Phase C we expected the fish to recuperate from the stressful conditions they had suffered in both tanks: C_5 from the suboptimal water quality due to the halting of water circulation, and in C_6 from both suboptimal water quality and exposure to MeHg. With no measurable effect on the Shannon entropy of the system's response during Phase A (exposure to Na_2SeO_3), it was assumed that changes on this parameter at the end of Phase B and beginning of Phase C would be mostly attributed to the effects of the water quality and of the exposure to MeHg, that the

effect of the latter should be much more noticeable than the former and that the initial exposure to Na_2SeO_3 would not bear on the results. The last assumption is important in order to compare cases C_5 and C_6, since both of them were made up of mixed populations, regarding Na_2SeO_3 treatment and the treatment might help the fish in their recuperation of homeostasis.

The working hypothesis in this recuperation Phase C was that upon both: improvement of the quality of the water and withdrawal of the MeHg from the water, the fish will initiate a period of gradual recuperation to reach homeostasis, that the recuperation will be faster in the group not treated with MeHg (i.e., in C_5) and that these changes will be reflected in the Shannon entropy of the shoal's centroid, i.e., that the Shannon entropy values would be outside the optimal operational point at the beginning of Phase C (due to the stressful conditions and MeHg poisoning) and would tend to the optimal operation point values identified in Chapter 5 for 26 (in C_5) and 19 (in C_6) fish as the systems approach homeostasis.

It has already been shown that MeHg-treatment had a measurable effect on the Shannon entropy of a European seabass biological system (Eguiraun et al., 2014) and Chapter 4. In the current work, immediately after the MeHg exposure period, the Shannon entropy values of the systems were of approximately 4 and 4.2 for C_5 and C_6 respectively. It is noteworthy that while in C_5 a more or less consistent trend towards increasing its Shannon entropy was observed during the subsequent recuperation period, such a trend was practically absent in C_6, with daily Shannon entropy values almost erratically oscillating between 4.8 and 4.1 as shown in Figure 6.2, i.e., the trend that the Shannon entropy followed during the recuperation Phase C was remarkably different in both cases, although their average values was not (Table 6.2), i.e. the trend had a discriminatory value, but the values themselves, individually or averaged, did not (Figure 6.2 and 6.3).

Table 6.2. Averaged Shannon entropy values in response to an event during the recuperation Phase C in cases C_5 (control) and C_6 (exposed to MeHg during the previous 15 days'period). Note also the different number of fish in C_5 and C_6.

	Shannon Entropy - Event
C_5 (26 fish)	4.4440±0.2964
C_6 (19 fish)	4.3799±0.3104

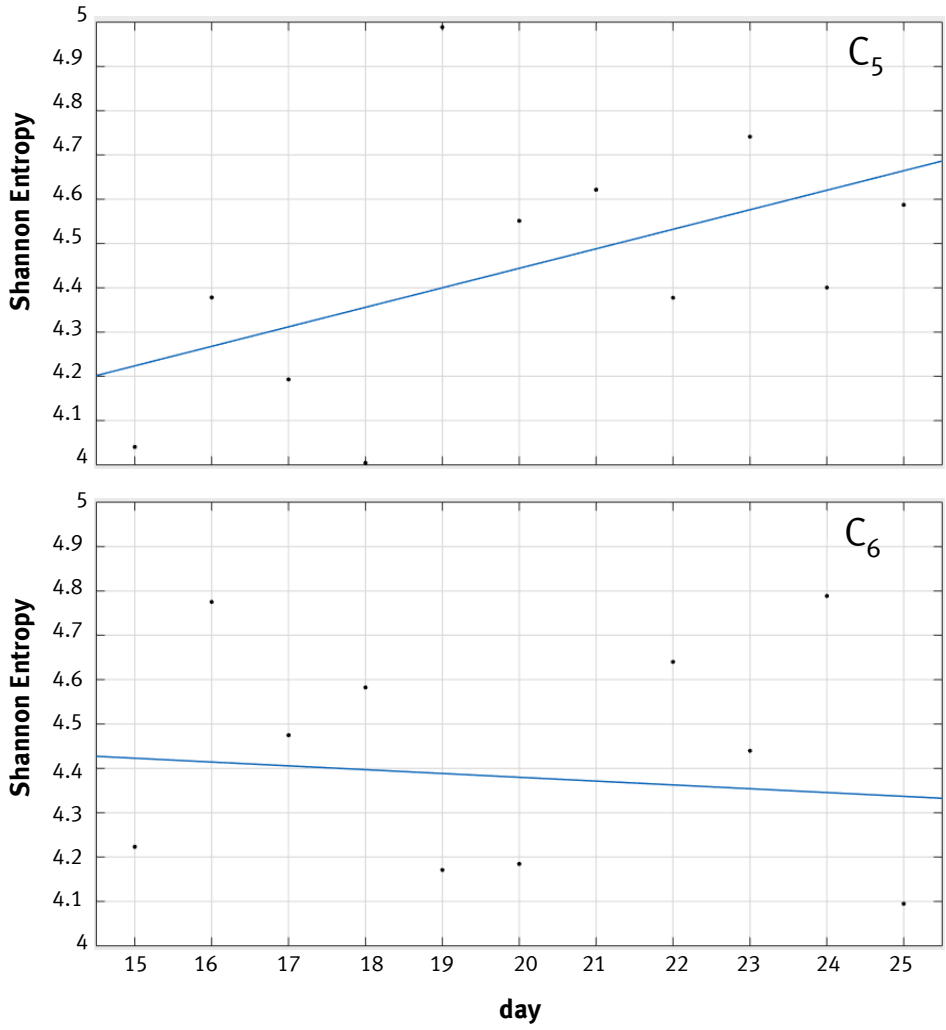

Figure 6.2. Evolution of the Shannon entropy of the fish system during the Phase C. C_5 (Control group with 26 fish) presents a more or less consistent trend towards increasing its Shannon entropy during the subsequent recuperation period. Such a trend was practically absent in C_6 (MeHg exposed group with 19 fish during the recovery period).

A second observation from this work is that 11 days may not be a sufficiently long period of time for the European seabass fish system to achieve a full recovery from MeHg contamination, probably reflected by the randomness of the daily oscillation in its Shannon entropy. Shannon entropy on the other hand did seemed to follow a clearly increasing trend when the system was recuperating from a more modest stress (see C_5 in Figure 6.2), were the lack of water circulation for 2 days periods may have only slightly affected the fish welfare.

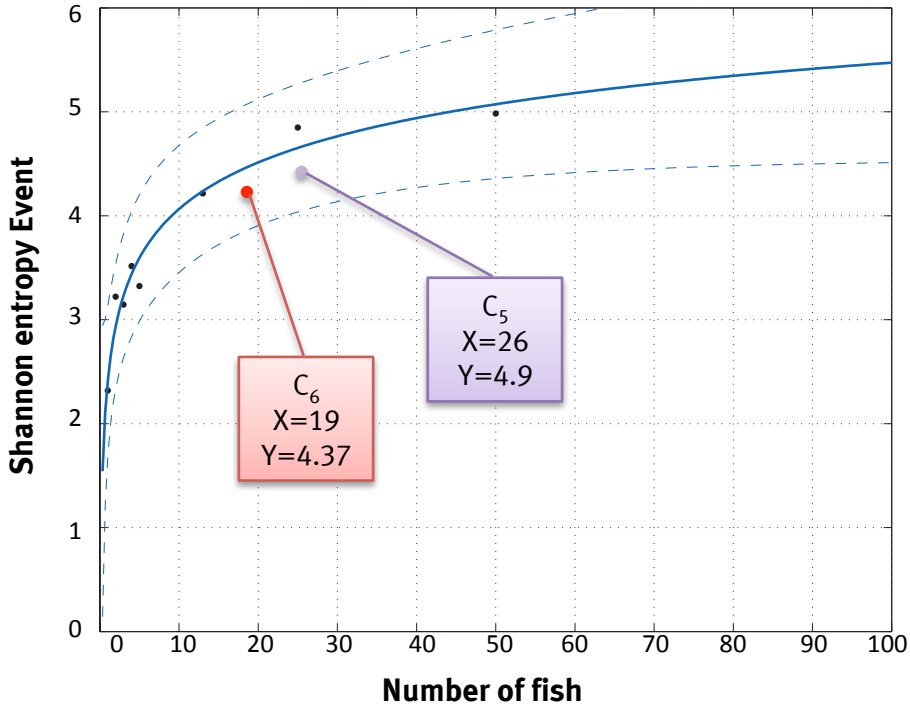

Figure 6.3. Averaged Shannon entropy values of C_5 (Control group of 26 fish, purple colour) and C_6 (MeHg-exposed group of 19 fish, red colour) as response to an event plotted in the curve obtained with the data obtained in Chapter 5.

With the current data, and taking into account that the experiment was not designed for this particular monitoring methods, it is remarkable that the tool designed rendered meaningful results that corroborate the results obtained when investigating the effect of the number of individuals (Chapter 5). It is tempting to speculate that the fish behave like a system that suffers an alteration when a perturbation is introduced and that, unless the perturbation has a very big impact (such as that of MeHg poisoning), the system gradually re-adapts itself and gets back to a steady state. However, when the perturbation introduced has major consequences, the system presents a much less clear evolution along the recovery period, as seen in C_6.

6.5. Overall conclusion

At this point, the tool designed and related techniques may have an application in the monitoring of fish welfare and seafood safety. Thus, fish might be considered not suitable for consumption if their Shannon entropy is not within the optimal working range and displays widely daily oscillation patterns. These results may also be a first step towards classifying different stressors on fish systems according to the responses they induce on their Shannon entropy as well as helping to verify the fish welfare as absence of stressors.

We can therefore conclude from this Chapter that the tool developed in Chapter 4 worked satisfactorily when applied to a complex, challenging, real-life experimental set up, and that it rendered meaningful results that sustain our initial hypothesis of its potential to function as an adequate monitoring tool in Fish Welfare Assurance System and Hazard Analysis and Critical Control Points systems.

This work tentatively confirms the proposed Hypothesis but additional experiments are required for a firm confirmation that exposure to MeHg would affect the entropy of the trajectory of the fish system and change its value, taking it outside what could be considered the optimal working range, and that removal of the contaminant would bring it back to the optimal working range.

CHAPTER 7
MODELLING

7.1. Introduction

This last chapter of the Thesis deals with using the knowledge generated in the earlier activities to create a first version of a model that ultimately will permit the integration of the Biological Warning System into a monitoring tool applicable to fish farming.

Two key issues in modelling are the complexity of the system subjected to study and the complexity of the model one wishes to create. Depending on those two degrees of complexity, different modelling techniques should be applied.

The different types of modelling methods have been described in the Chapter 2 of the present Thesis. For the challenge this Thesis faces, the most suitable approach is the so called "Black box" modelling, a widely used technique for approximating unknown systems (Golubev and Horowitz, 1982; Kalaitzakis et al., 2008; Ma and Zuo, 2014): in the system dealt with in the present Thesis only their inputs and outputs are known, there is no physical knowledge about the system, and the knowledge obtained is calculated from data acquired by observation/experimentation of/on the system, i.e., there was no previous knowledge about the European seabass system and the data input-output relationships were established and the model built based on the experimental cases performed in the previous chapters (Chapters 4-6).

7.2. Parameters of the model

As already mentioned, three sub-models have been created using knowledge extracted from the activities described in Chapters 4 to 6. Subsequently, these sub-models should be combined to generate the integrated, or "overall" model. Basically, the present model is designed to detect differences in the status of the fish and generate the corresponding warning signals between the real online monitoring inputs and those established as "normal" inputs (based on knowledge) for the desired optimal operation point for the fish system.

Two of the reasons that make the modelling of the present system particularly complex are, on one hand, that there is no previous knowledge of the physical nature of the fish system itself and, on the other, that it bears a series of inherent attributes which are fundamentally complicated to model:

- ***Non-linear:*** basically, a non-linear system's outputs do not obey the superposition principle, thus the outputs are not a linear combination of the inputs. This means that, since the non-linear equations from these systems are very difficult to solve, these systems are usually linearized around a certain operation point.
- ***Dynamic and time variant:*** since the fish system varies and evolves with time.
- ***Stiff:*** related with the system dynamics, systems that merge slow and fast

dynamics are considered to be stiff. These two different characteristics make the system work in very distant working points, which makes it a difficult system to deal with. For example, in the present Thesis there are very different time scales because there can occur simultaneously events in a time scale of seconds (i.e., the hit in the tank) and in a scale of days or weeks (the presence of contaminants).

- ***With initial conditions not equal to zero:*** this applies to systems that present a previous history, which affects directly to its evolution. Sometimes, the previous history is unknown.

As already mentioned, a black box modelling technique grounded on the input-output data relationship helped to deal with these limitations, and in this case, the input-output parameters used were those empirically obtained in the previous Chapters, namely: the fish number, and the system's Shannon entropy in a basal state, as a response to an event, and its daily evolution.

7.3. Construction of the model

A schematic representation of a model integrating the three sub-models created based on empirical data is shown in Figure 7.1. The three sub-models are:

- Basal reference sub-model: built using the "basal entropy", i.e. the entropy generated by the fish system in its basal state.
- Event reference sub-model: built using the "event entropy", i.e. the entropy of the trajectory generated by the fish system in response to a hit in the tank.
- Basal/Event relationship reference sub-model: built using the ratio between the "basal" and the "event" entropies.

The error signals created measure the difference between the desired behaviour of the system from an optimal point of view and the online signals measured by the monitoring tool. These error signals are the outputs of the proposed "overall" model and they should be integrated in knowledge models of higher order, i.e., as inputs to the "Model integration" block in Figure. 7.1.

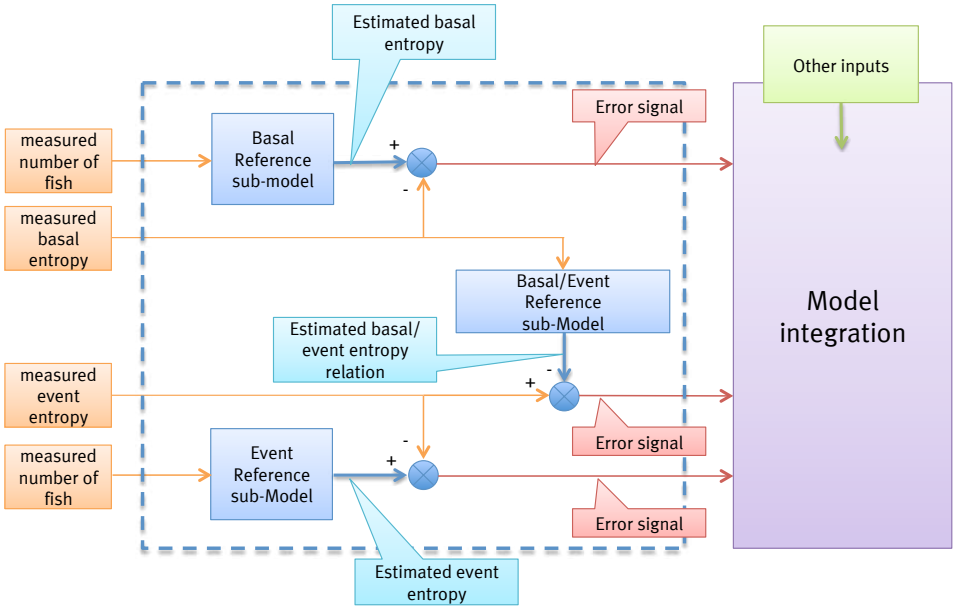

Figure 7.1. Schematic representation of the model defining inputs, generated outputs due to error signals, internal variables and sub-model interactions. The output error signals should feed the subsequent phase of the model where all this information is integrated represented by the Model integration box.

7.3.1. Basal-state reference sub-models

The sub-models corresponding to the reference basal states were built using the data obtained in Chapters 5 and 6 corresponding to the Shannon entropy of the basal states and their evolution during 5 days for the different number of fish tested (1, 2, 3, 4, 5, 13, 25 and 50 from Chapter 5 and 76 from Chapter 6). Table 7.1 summarizes the daily mean values (each day 3 measurements of the basal entropy were made) of the Shannon entropy of the basal state.

Table 7.1. Shannon entropy values of the basal states. The data in the table were obtained from the experiment described in Chapter 5, except those denoted with *, which came from the experiment described in Chapter 6.

# fish	Day				
	1	2	3	4	5
1	0.56±0.29	1.28±0.74	0.68±0.59	1.37± 0.37	1.44±0.70
2	2.36±0.23	2.36±0.23	2.36±0.23	2.36±0.23	2.36±0.23
3	2.96±0.26	2.96±0.26	2.96±0.26	2.96±0.26	2.96±0.26
4	3.46±0.35	3.46±0.35	3.46±0.35	3.46±0.35	3.46±0.35
5	3.55±0.35	3.55±0.35	3.55±0.35	3.55±0.35	3.55±0.35
13	4.04±0.20	4.02±0.15	4.48±0.45	4.10±0.25	4.16±0.41
25	4.73±0.04	4.63±0.23	4.58±0.15	4.49±0.21	4.58±0.27
50	4.77±0.19	4.80±0.22	4.67±0.10	4.71±0.11	4.88±0.30
76*	4.57±0.214	4.33±0.30	4.71±0.06	4.79±0.67	4.61±0.45

The data from Table 7.1 were used to construct the daily sub-models corresponding to the basal states for each of the 5 days by fitting a curve that followed the equation: $y=u\cdot x^{v}+w$, as it was done in Chapter 5, where y is the Shannon entropy; x is the number of fish and u, v and w are the coefficients of the fitted curve. The goodness of the fit for each curve was also calculated. The data are shown in Table 7.2 and plotted in Figure 7.2.

Table 7.2. Parameters of the sub-models for the daily basal states estimated using the data from Table 7.1.

$y=u\cdot x^v+w$		1 day	2 day	3 day	4 day	5 day
Model name		**basal 1**	**basal 2**	**basal 3**	**basal 4**	**basal 5**
Coefficients	***u***	-4.32	-3.694	-4.302	-3.896	-3.879
	v	-0.7532	-0.6116	-0.7349	-0.5099	-0.5076
	w	4.887	4.917	4.957	5.229	5.248
Goodness of the fit	**SSE**	0.1509	0.2686	0.05532	0.05245	0.1316
	R-Square	0.9898	0.9743	0.9962	0.995	0.9875
	Adjusted R-Square	0.9864	0.9657	0.9949	0.9934	0.9833
	RMSE	0.1586	0.2116	0.09602	0.0935	0.1481

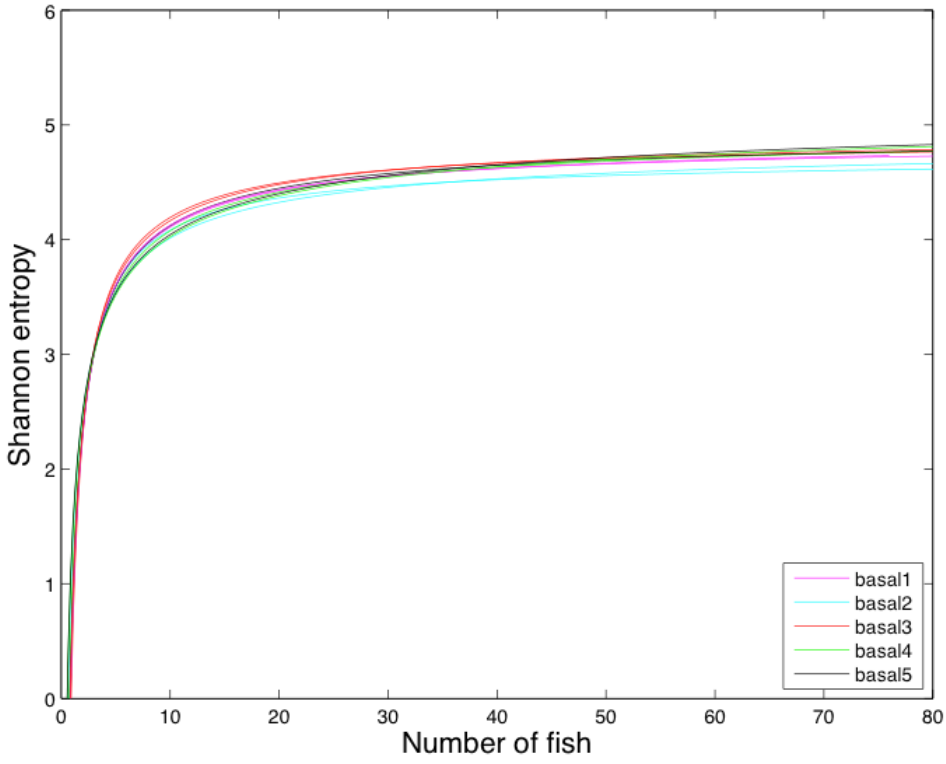

Figure 7.2. Plot of the daily basal sub-models (basal 1, 2, 3, 4 and 5 as named in Table 7.2) that correspond to the Shannon entropy of the basal states of days 1 to 5 respectively.

7.3.2. Response to the stochastic event reference sub-models

The sub-models corresponding to the reference responses to the stochastic event were also built using the data obtained in Chapter 5 - 6 corresponding to the Shannon entropy of the response to the event and their evolution during 5 days for the different number of fish tested (1, 2, 3, 4, 5, 13, 25 and 50 from Chapter 5 and 76 from Chapter 6). Table 7.3 summarizes the daily values (in this case, only one measurement per day was made) of the Shannon entropy of the response to the event state.

Table 7.3. Shannon entropy values of the response to the event. The data in the table were obtained from the experiment described in Chapter 5, except those denoted with *, which came from the experiment described in Chapter 6.

# fish	Day				
	1	2	3	4	5
1	2.78	2.13	2.16	1.82	1.90
2	3.22	3.22	3.22	3.22	3.22
3	3.15	3.15	3.15	3.15	3.15
4	3.51	3.51	3.51	3.51	3.51
5	3.32	3.32	3.32	3.32	3.32
13	4.37	4.14	4.30	4.04	4.23
25	4.91	4.65	4.57	4.67	5.43
50	4.89	5.45	4.95	4.81	4.82
76*	5.16	4.74	4.78	5.86	5.29

The data from Table 7.3 were used to construct the daily sub-models corresponding to the response to the event states for each of the 5 days by fitting a curve that followed the equation: $y=u\cdot x^{v}+w$, as it was done in Chapter 5, where y is the Shannon entropy; x is the number of fish and u, v and w are the coefficients of the fitted curve. The goodness of the fit for each curve was also calculated. The data are shown in Table 7.4 and plotted in Figure 7.3.

Table 7.4. Parameters of the sub-models for the daily response to the event estimated using the data from Table 7.3.

$y=u\cdot x^v+w$		1 day	2 day	3 day	4 day	5 day
Model name		**event 1**	**event 2**	**event 3**	**event 4**	**event 5**
Coefficients	***u***	-613.5	-4.899	-3.854	-14.55	-4.75
	v	-0.000968	-0.2056	-0.2775	-0.05944	-0.2733
	w	616.2	7.133	6.095	16.72	6.756
Goodness of the fit	**SSE**	0.3123	0.6407	0.2658	0.7401	0.8592
	R-Square	0.9526	0.9229	0.9613	0.9329	0.9199
	Adjusted R-Square	0.9368	0.8972	0.9483	0.9105	0.8933
	RMSE	0.2281	0.3268	0.2105	0.3512	0.3784

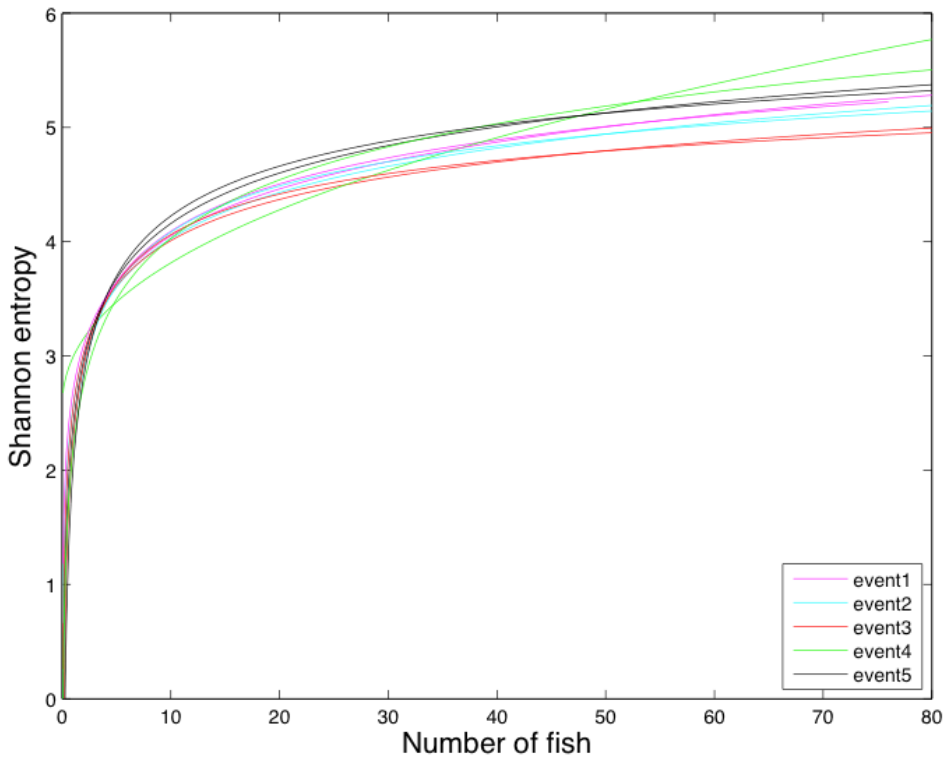

Figure 7.3. Plot of the daily response to the event sub-models (event 1, 2, 3, 4 and 5 as named in Table 7.4) that correspond to the Shannon entropy of the response to the event for days 1 to 5 respectively.

7.3.3. Basal/Event response reference sub-models

A third model was constructed based on the existing relationship between the Shannon entropies of the basal state and that of the response to the event. This model uses as input the mean of the basal Shannon entropies and as output the Shannon entropies of the responses to the event for each of the 5 days. Table 7.5 shows these values, which are obtained by merging Table 7.1 and Table 7.3. Once again, all the data belong to Chapter 5, except by the one corresponding to 76 fish that belong to Chapter 6.

Table 7.5. Merging of Tables 7.1 and 7.3 showing the Shannon entropy values for the basal state and for the response to event for each experimental day. The data in the table were obtained from the experiment described in Chapter 5, except those denoted with *, which came from the experiment described in Chapter 6.

# fish	1 day		2 day		3 day		4 day		5 day	
	Basal	Event	Basal	Event	Basal	Event	Basal	Event	Basal	Event
1	0.56	2.78	1.28	2.13	0.68	2.16	1.37	1.82	1.44	1.90
2	2.36	3.22	2.36	3.22	2.36	3.22	2.36	3.22	2.36	3.22
3	2.96	3.15	2.96	3.15	2.96	3.15	2.96	3.15	2.96	3.15
4	3.46	3.51	3.46	3.51	3.46	3.51	3.46	3.51	3.46	3.51
5	3.55	3.32	3.55	3.32	3.55	3.32	3.55	3.32	3.55	3.32
13	4.04	4.37	4.02	4.14	4.48	4.30	4.10	4.04	4.16	4.23
25	4.73	4.91	4.63	4.65	4.58	4.57	4.49	4.67	4.58	5.43
50	4.77	4.89	4.80	5.45	4.67	4.95	4.71	4.81	4.88	4.82
76*	4.57	5.16	4.33	4.74	4.71	4.78	4.79	5.86	4.61	5.29

A linear relationship was identified between the Shannon entropy value of the basal states and that of their corresponding responses to the stochastic event, i.e. the model fitted a first order polynomial described by the equation: $y=u\cdot x+v$, where y is the Shannon entropy of the response to the event, x is the basal Shannon entropy and u and v are the coefficients of the fitted curve. The goodness of the fit for each curve was computed and all the values are sumarized in Table 7.6 and plotted in Figure 7.4.

Table 7.6. Parameters of the sub-models for the relationship between the daily Shannon entropies of the basal state and the corresponding response to the event response estimated using the data from Table 7.5.

$y=u\cdot x^v+w$		1 day	2 day	3 day	4 day	5 day
Model name		**basal-event 1**	**basal-event 2**	**basal-event 3**	**basal-event 4**	**basal-event 5**
Coefficients	***u***	0.5805	0.8403	0.6516	0.952	0.9462
	v	1.924	0.8816	1.496	0.459	0.511
Goodness of the fit	**SSE**	1.595	0.9456	0.6939	1.45	1.301
	R-Square	0.7578	0.8863	0.8989	0.8686	0.8788
	Adjusted R-square	0.7232	0.87	0.8844	0.8498	0.8615
	RMSE	0.4774	0.3675	0.3148	0.4551	0.4311

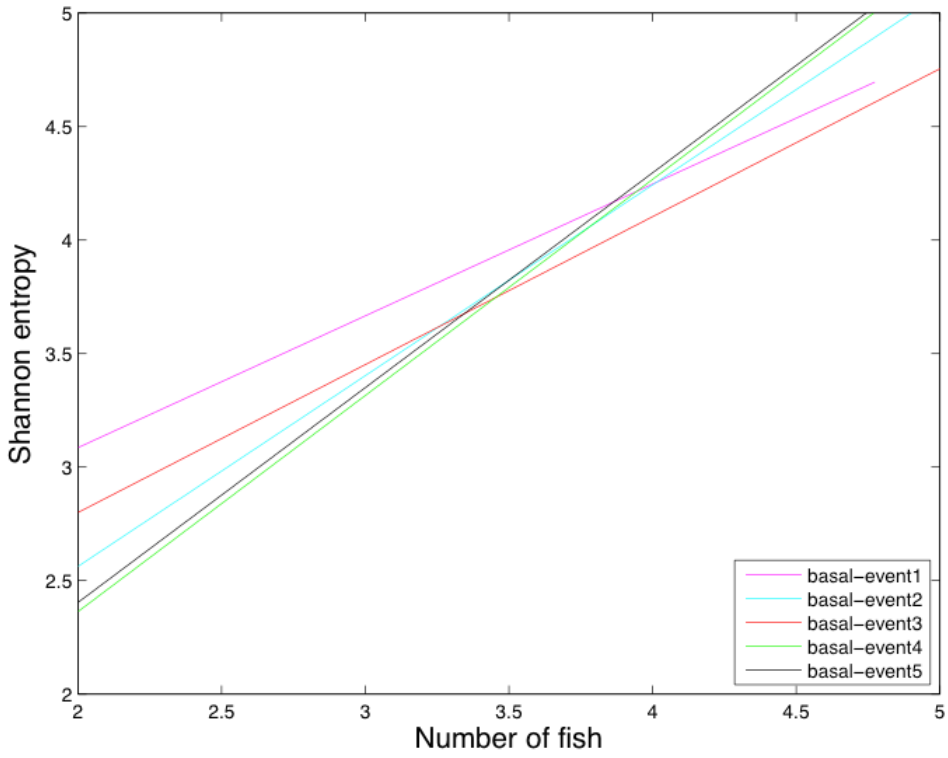

Figure 7.4. Plot of the relationship between the basal and the response to the event entropies for each experimental day. The name of the sub-models: basal-event 1, 2, 3, 4 and 5 are described in Table 7.5.

7.4. Integrated model

Merging the previously defined sub-models within the architecture shown in Figure 7.1, the overall model proposed is summarized in Figures 7.5, 7.6 and 7.7; and the elements of the model are explained in Table 7.7. The main purpose of this model is to serve as a reference point to detect deviations from the expected Shannon entropy values. For a system operating in a satisfactory manner, the outputs of each sub-model would be within their acceptable operational range, i.e., within the –empirically in this case– established critical limits. Shannon entropy values outside these limits would indicate that a deviation from the acceptable operational range has taken place and immediately a pre-established protocol must be followed until the system is shown to be restored to its acceptable operational range.

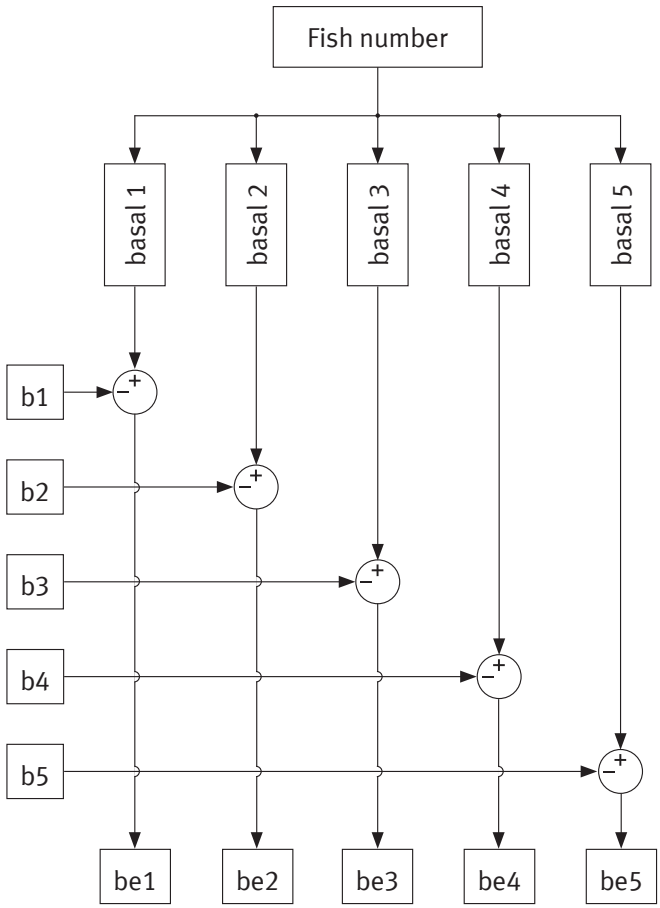

Figure 7.5. Reference sub-model based on the evolution of the basal Shannon entropy of the system during 5 consecutive days. The measured Shannon entropy of the basal state is compared with the empirically estimated to be the optimal one, and the difference between the two (the error) is calculated.

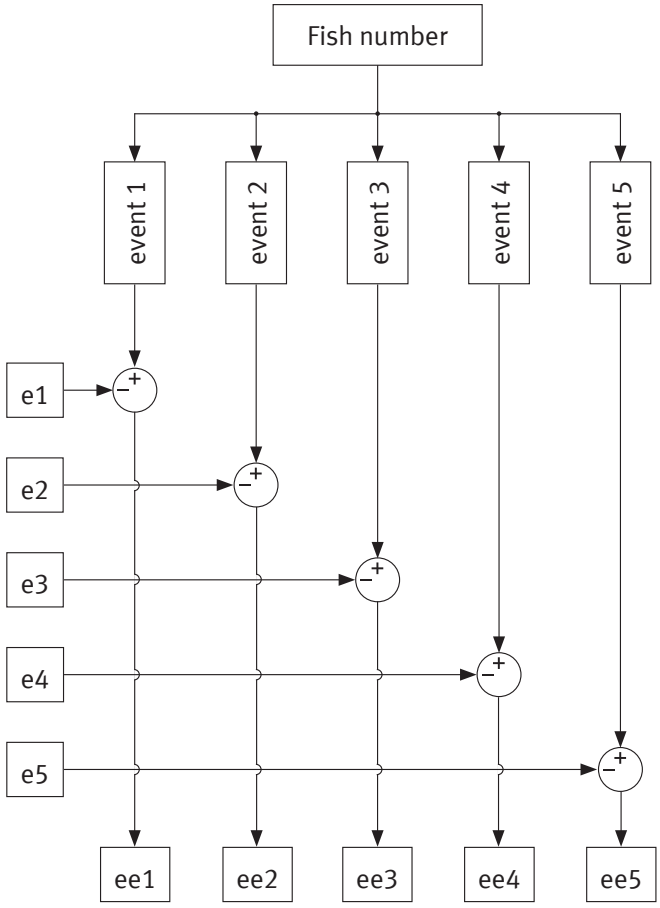

Figure 7.6. Reference sub-model based on the evolution of the Shannon entropy corresponding to the system's response to the stochastic event during 5 consecutive days. The measured Shannon entropy of the response to the event is compared with the empirically estimated to be the optimal one and the difference between the two of them (the error) is calculated.

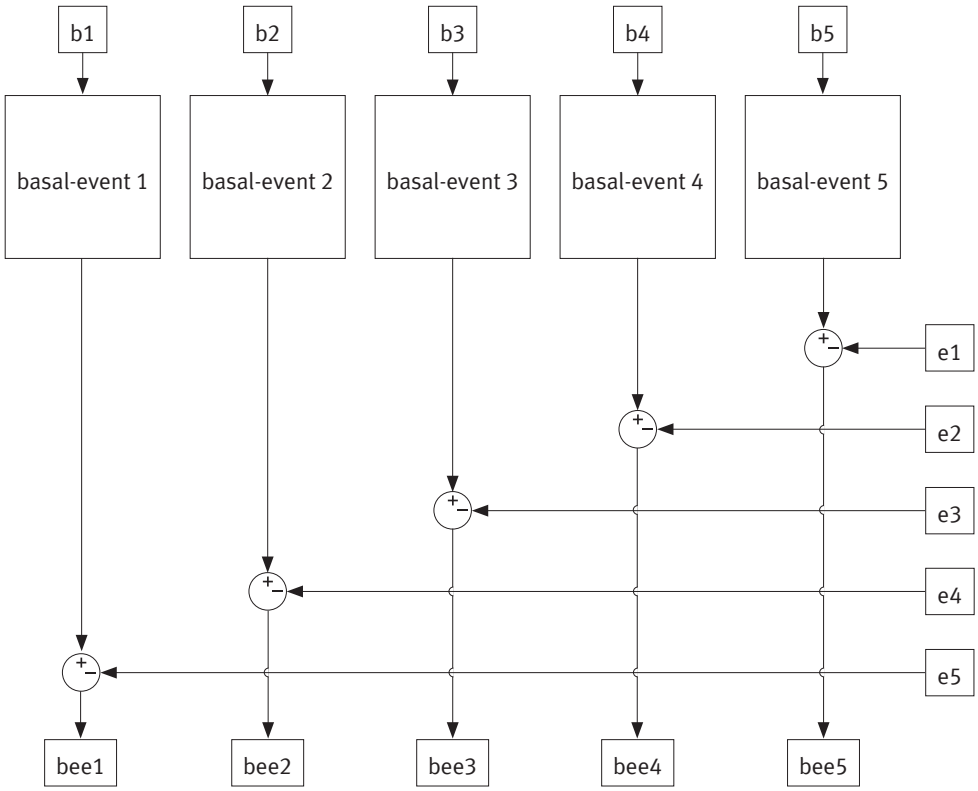

Figure 7.7. Basal-Event Reference sub-model based on the evolution of the Shannon entropy corresponding to the system's response to the stochastic event during 5 consecutive days. The calculated ratio of the Shannon entropies is compared with the empirically estimated to be the optimal one and the difference between the two of them (the error) is calculated.

Table 7.7. Elements of the model, shown as abbreviations in Figures 7.5, 7.6 and 7.7.

Parameter	Variable	Type
Number of fish	Number of fish introduced as a set point	Input-Set value
b1…b5	Shannon entropy of the system in the basal state	Input-variable
e1…e5	Shannon entropy of the system in response to the stochastic event	Input-variable
be1…be5	Difference between the established acceptable basal Shannon entropy of the system and the measured one	Output-variable
ee1…ee5	Difference between the established acceptable Shannon entropy of the system as a response to a stochastic event and the measured one	Output-variable
bee1…bee5	Difference between the acceptable and the measured ratios between the basal Shannon entropy of the system and the Shannon entropy in response to the stochastic event	Output-variable
basal1…basal5	Sub-model corresponding to the Shannon entropy of the system in the basal state	Model-internal
event1…event5	Sub-model corresponding to the Shannon entropy of the system in response to the stochastic event	Model-internal
basal-event1 … basal-event5	Sub-model corresponding to the difference between the acceptable and the measured ratios between the basal Shannon entropy of the system and the Shannon entropy in response to the stochastic event	Model-internal

7.5. Discussion

The introduction in fish biological systems of factors that affect fish health and welfare (such as methylmercury, chlorine, and others) have been shown by several authors to induce changes in the entropy of the system (Bae and Park, 2014; Forlim and Pinto, 2014; Kadota et al., 2011; Liu et al., 2011a; Quach et al., 2013; Spasic et al., 2011). These variations in the Shannon entropy could serve the fish farmer to detect alterations/malfunctions, proceed immediately (following standard operation procedures that must be defined by each enterprise) to identify the cause of the deviation and to eliminate it or bring it under control. In some cases, these actions will prevent a contaminated batch to reach the seafood market, in others, it will aid in the early detection of alterations that could carry large financial losses if left unattended (such as diseases, presence of parasites, aggression) and, in all of them, it will help to keep the system's homeostasis and avoid endangering the welfare of the animals.

Modelling biological systems is a complex matter, not only due to their non-linearity, the fact that they change and evolve with time, merging slow and fast dynamics and with initial conditions different from zero, but also because the total number of variables/properties that make up the system, their combinations and interactions are often unknown. To establish a functional Biological Warning System in fish farming means that relevant information must be collected and included in the main model. For example, the genetic make up of the species may determine the dynamics of their responses, swimming and shoaling behaviour (Wark et al., 2011). Different contaminants may affect the behaviour in different manners (Brodin et al., 2013; Eguiraun et al., 2014) and it is also possible that the effect changes with the length of the exposition time.

It must be kept in mind that in a stiff system, the fast responses may correspond to a normal response, for example to a stressor (a perceived attack, a hit in the tank). However it is the stressors that take place in a longer time scale (days, weeks and/or months) the ones that negatively affect the welfare of the fish increasing their susceptibility to diseases and therefore bear a higher relevance within the model due to their higher capability to seriously disrupt the homeostasis of the entire system.

In summary, this model must be considered as a first step in achieving an on-line fish welfare monitoring method. Further work needs to be done in order to improve and optimize the proposed model and/or in order to create new ones. For example, there maybe factors affecting fish welfare that need a different modelling approach. The improvement of the model demands the collection and integration into it of all relevant information; testing and validating it for different settings and factors affecting fish welfare.

CHAPTER 8
GENERAL DISCUSSION, CONCLUSIONS, THESIS AND FUTURE PROSPECTS

This Chapter summarizes the contributions the present Thesis has made to the state of the art on the development of monitoring tools based on Biological Warning Systems applicable to aquaculture. The first part of this Chapter consists of a General Discussion, followed by the Conclusions and the Thesis of the study. The last section is devoted to some suggestions about future possibilities in this research area.

8.1. General discussion

The Main Objective of the Thesis was to design and develop a tool that using a fish system as a sensor could be implemented within a Biological Warning System in the aquaculture industry. European seabass (*Dicentrarchus labrax*) was selected as the model species for its relevance in Southern European aquaculture. It should be stressed that the aim was not to map behavioural characteristics such as time swimming or resting, aggressive behaviour or the shoaling itself, which would require a different methodological approach.

The Main Objective was targeted in **Chapter 4** that describes the structure of the tool. The information was non-invasively acquired by video recording the trajectory described by the shoal of fish as a response to a stochastic event (a hit in the tank) and it was further processed by different non-linear signal processing algorithms, of which the Shannon entropy of the system was selected for being the most diagnostic one to discriminate between groups known to be very different. The selection of Shannon entropy as a suitable parameter is in accordance with the work of Wark et al. (2011) on stickelbacks. Interestingly, these authors showed that the Shannon entropy was able to extract information from the system that classical analysis tools did not and, in addition, demonstrated that the shoaling behaviour of the stickelbacks had a clear genetic component.

The non-invasive tool developed targeted the responses of the fish groups rather than that of individual fish, both to reduce the computational effort and because the response of the group may be considered the result of integrating all the responses contributed by each individual fish, and therefore may represent better the system than that of individual fish, since the latter may be influenced by the physiological status of the individual, its size, status in the school's hierarchy and other factors that are usually unknown when the monitoring is performed. Also, the response to a stochastic event was measured instead of other behavioural aspects (swimming pattern, daily activity, feeding, aggressiveness, etc.) because it permits to restrict the computational analysis in time to the duration of the response (three minutes was sufficient in our case, rather than observing the animals for longer periods of time where more variables may play a role) and to reproduce the event at will in other settings for comparison purposes. **Chapter 4,** thus, answers **Research Questions 1** and **2**, demonstrating that fish subjected to a perturbation/stressor alter their behaviour and that the differences are measurable in a non-invasive manner.

The number of fish is a particularly interesting variable when trying to establish the Optimal Working Point/Range for the system because it had not been tested how

it could affect the system's behaviour, particularly for shoaling species. This leads to the work performed in **Chapter 5,** that targets the **First Secondary Objective**, namely how the fish system reacts to a change in the number of individuals and also answers the **3rd Research Question**, indicating that the number of fish is indeed a variable that influences the Shannon entropy of the system.

The number of fish is usually not considered a variable influencing the system unless the number is too high, for example during crowding, which is known to negatively affect the fish welfare in a species-specific manner (Boerrigter, 2015). Based on previous studies (Di Marco et al., 2008; Papoutsoglou et al., 1998) it was not expected to find large differences attributable solely to the number of fish, for example between the groups with 81 and 41 fish used in **Chapter 4**. Nevertheless, since this was a variable that had not been tested before, it was considered interesting to assess its weight in the Shannon entropy of the system.

Chapter 5 showed that the Shannon entropy of the European seabass system is highly dependent on the number of individuals for a few fish (from 1 to 5) becoming more independent from the number as it increases and that this dependence nicely fits an exponential curve. Nonetheless, it must be mentioned that the physiological meaning of this dependency remains unknown and therefore it cannot be said that it represents a response to a stress, for example that too few individuals may be considered a stressful situation for a shoaling species, although in view of the shoaling nature of the species this may well be the case. It was also observed that the Shannon entropy of the response to an event of the fish system was usually higher than that of its basal state.

For European seabass monitoring it seems that a number between 5 and 13 individuals may be the lowest suitable number to achieve meaningful results —for example to perform experiments or to set up a Biological Warning System monitoring unit. However, there is a need to identify also the number corresponding to *too many individuals*, i.e. the number of individuals that would make the system collapse. For example, overcrowding may limit the space where each animal can move, which will in turn make the movement of the centroid of the shoal appear increasingly stagnatic, regardless of whether the individuals themselves move or not. In this latter case the Shannon entropy value of the system may revert to lower values, or even become zero for a completely static centroid. Thus, it is necessary to identify the values of both *many individuals* and *too many individuals* within which the results are valid, i.e., within the Operational Working Range of the system. For the applications targeted by this work, i.e., implementation of a Biological Warning System in aquaculture and for experiments that require fish, we consider that 5-13 fish may be adequate using the same species and strains of fish, tanks and conditions used in this Thesis.

The concepts shown in **Chapter 4** and **5** may probably apply not only to European seabass, but also to other similarly shoaling species. Although the behaviour and response of the system will likely be species-specific (Boerrigter, 2015), the author considers that this approach might be applied with few modifications to monitoring salmon, charr, cod or trout, but it will likely need some modifications if applied to species with different behaviour, such as eels or flatfish.

The application of the tool to an experimental case where European seabass were exposed to sodium selenite and/or methylmercury is shown in **Chapter 6**. This Chapter addresses the **2nd and 3rd Secondary Objectives**, namely, the reaction of the fish system to a dietary supplement of selenium and to methylmercury, a neurotoxic contaminant and responds to **Research Questions 1** and **2**, showing that fish subjected to a chemical contaminant do alter their behaviour and that it is possible to non-invasively quantify it. Moreover, **Chapter 6** uses the knowledge generated in **Chapter 5** on the influence of the number of fish on the Shannon entropy of the system.

The study of **Chapter 6** was a complicated one because the addition of sodium selenite and/or methylmercury to the water in the tanks involved a halting of the water circulation for a few days and although the water quality parameters were within acceptable limits, some turbidity developed that possibly affected the welfare of the fish and the Shannon entropy of the system's response. However these were limitations imposed by the experiment that can, in fact, resemble real life conditions and were therefore very interesting to test. The addition of sodium selenite, a dietary supplement (used also for humans), for a week did not affect the Shannon entropy of the system. This was expected because the amount added was within the limits considered to have a counteracting effect on methylmercury toxicity on the fish system. In was noteworthy that the values of Shannon entropy for the fish system during the entire week agreed with those calculated according to the equation of the fitted curve from **Chapter 5**.

Unfortunately, the quality of the images obtained during the period of exposure to the environmental contaminant methylmercury was too poor to produce reliable results. However, biochemical analyses indicated that methylmercury-treatment had had a clear negative effect, and that sodium selenite had exerted the expected protective effect (Vitalle, 2014).

The last phase of **Chapter 6** indicates that the Shannon entropy of the fish system during an 11 day recovery period after the exposure to methylmercury lacked a clear tendency, displaying random and erratic values that changed from day to day, while it tended to increase in the not-exposed control group. This work presents two interesting implications: one is that 11 days may not a be a sufficiently long period of time for the European seabass fish system to achieve a full recovery from the amount of methylmercury used in this work, and the other is that, if one wishes to use the Shannon entropy as a parameter, both its value and the daily evolution of the value, must be taken into account for a fish welfare assessment system.

The last Chapter of this Thesis, **Chapter 7**, consists of developing a model based on the knowledge generated in **Chapters 4-6**, and thus fullfils the **3rd** and last **Objective** of the Thesis and responds to the **4th Research Question**, by building a mathematical model that responds to differences between the measured and the desired behaviours of the system.

Modelling biological systems is a complex matter, not only due to their non-linearity, the fact that they change and evolve with time, merging slow and fast dynamics and with initial conditions different from zero, but also because the total number of variables/properties that make up the system, their combinations and interactions are often unknown. Based on the empirical results obtained it was considered necessary that the model should contain at least three reference sub-models, namely a basal sub-model, a sub-model on the response to the event and the basal-event relation sub-model. Moreover, the model should register the response of the system during at least five consecutive days based on the information obtained in Chapter 6, namely that systems subject to some stressors (methylmercury) may need some days to recover and that the daily evolution of the Shannon entropy is therefore a meaningful parameter. To establish a functional Biological Warning System in fish farming means that relevant information must be collected and included in the main model. For example, the genetic makeup of the species may determine the dynamics of their responses, swimming and shoaling behaviour (Wark et al., 2011). Different contaminants may affect the behaviour in different manners (Brodin et al., 2013; Eguiraun et al., 2014) and it is also possible that the effect of the stimuli/stressor change with the length of the exposition time, perhaps due to adaptation of the system to the stimuli (for example to lack of exercise, poor diet, etc) or to the stimuli inducing a final collapse of the system (for example high mortality due to diseases and infections). Furthermore, different species will likely respond in a different manner to the same stressor, as shown by the physiological parameters measured in catfish and eel in response to the stress generated during transport (Boerrigter, 2015).

In any case, it is clear to the author that the knowledge and the tool generated in this work is a valid addition and complement to the wealth of knowledge generated from studies on biochemical, histological and physiological parameters and particularly on works applying -omics techniques, to obtain an integrated vision of what fish welfare really is, the physiological meaning of the variations in the Shannon entropy of the system, and ultimately to achieve an effective, affordable and reliable on-line tool for welfare monitoring.

8.2. Conclusions

1. A monitoring tool has been developed consisting of a methodology for image acquisition, processing and non-linear trajectory analysis of the fish shoal's cluster centroid, suitable to identify variations in the response of a fish group to an event.
2. The Shannon entropy of the fish system has been shown to be the best parameter to analyse the trajectory of the cluster's centroid.
3. The Shannon entropy of the European seabass system is highly dependent on the number of individuals for a few fish (from 1 to 5) becoming more independent from the number as it increases.

4. The relationship between the Shannon entropy of a European seabass system and the number of fish fits an exponential curve.
5. The tool developed worked satisfactorily when applied to a complex, challenging, real-life experimental set up, and it rendered meaningful results that sustain the initial hypothesis of this Thesis.
6. The tool indicated that the addition of sodium selenite for six days to the European seabass system in concentrations expected to exert a protective effect against methylmercury toxicity did not affect the Shannon entropy of the system.
7. The tool indicated that the addition of methylmercury for two weeks to the European seabass system in concentrations expected to exert a toxic effect did affect the Shannon entropy of the system.
8. Application of the tool to a European seabass system indicated that not only the Shannon entropy value but also its daily evolution need to be included as parameters in a fish welfare monitoring procedure.
9. A model has been developed integrating the daily behaviour of three reference sub-models, namely the basal state reference sub-model, the response to the stochastic event reference sub-model and the basal-event response reference sub-model.
10. The tool, after the necessary improvements and optimizations, has the potential to be embedded in an on-line/real time architecture to monitor fish schools in a farm and in the wild, and therefore this kind of approach may find an application as a monitoring tool in Fish Welfare Assurance and Hazard Analysis and Critical Control Points systems in fish farming, and to identify contaminated waters in environmental monitoring programs.

8.3. Thesis

The Thesis of this study is that a fish system can be used as a biological sensor because the alteration of its behaviour in response to external stimuli is quantifiable and can be non-invasively monitored. Furthermore, the alteration of the behaviour, as measured by the Shannon entropy of the system, has the potential to serve as a tool for on-line fish welfare monitoring.

8.4. Future prospects

Given the increasing interest in setting up Recirculating Aquaculture Systems where monitoring the entire production unit (i.e. the tank) may become feasible, the methodology here proposed may have a clear potential to aid implementing intelligent monitoring and control aquaculture systems but it needs to be tested and validated

with more contaminants, stressors and fish species, prior to be embedded in real-time automatic systems using artificial intelligence methods. Technically, the method presented here demands a relatively large computation capability, particularly for the image-processing step, which is certainly susceptible of improvement. Therefore a future line of research may deal with the improvement of the methodology to obtain information on the analysis of the fish clusters trajectories; for example technically improving the data acquisition tools, such as echo-sounds, infrared images and hyperspectral images, among others.

This approach also demands the identification of the *too many fish number* that would make the system collapse, not only because the measurements obtained outside that range would not be reliable, but also because it will probably indicate a loss of welfare due to crowding (which, as mentioned above, will also be a species-specific parameter, since some species thrive at very high densities). Many other variables will likely impact the system such as the size/weight of the fish, the degree of sexual maturation, genetic makeup and others.

A wide field of research lays ahead when exploring the possibilities of applying this and related tools to different fish species, from fresh- and seawater under different environmental conditions. And yet this work must be done if such a tool is to be implemented, particularly if it is intended to become a commercial technology or a product.

Additional research is required to optimize this kind of methodology in order to embed it within an on-line/real time architecture to monitor fish schools in a farm and in the wild, so that this kind of approach finds an application within Fish Welfare Assurance and Hazard Analysis and Critical Control Points systems in fish farming, and to identify contaminated waters in environmental monitoring programs.

Finally, the knowledge and the tool generated in this work must be merged to the wealth of knowledge generated from studies on biochemical, histological and physiological parameters and particularly on works applying -omics techniques, to obtain an integrated vision of what fish welfare really is, the physiological meaning of the variations in the Shannon entropy of the system, and ultimately to achieve an effective, affordable and reliable on-line tool for welfare monitoring.

LABURPENA
(SUMMARY IN BASQUE)

Sarrera

Etorkizun hurbilera begira gizakion kopurua ez ezik (Gerland et al., 2014; United Nations, 2014), itsas elikagaien ekoizpenaren eta kontsumoaren hazkundea espero da. Azken honen hazkundeak, batipat, akuikulturak bideratuko du (German Advisory Council on Global Change - WBGU, 2013). Akuikultura, ekoizpena handiagotzeko helburuagaz, itsas organismoen hazkunde kontrolatua da. Bertatik lortzen diren produktuak era askotakoak izan daitezke: arrainak, moluskuak, krustazeoak, algak eta itsas landareak dira ezagunenak. Baina kokodriloak, dortokak eta beste motatako zenbait animalia urlehortarrak ere ekoizten dira. Era berean, akuikultura instalazioek kokapen desberdinak euki ditzakete: itsas kostaldean zein itsasadarretan baina lur barnean zein ibai edo lakuen ondoan ere aurkitu daitezke.

Beste edozein ekoizpen prozesu bezala, sortzen diren produktuen kalitatea erronka garrantzitsuenetariko bat da. Eta kalitatea, kontuan izanda sortzen diren produktuak gizakion kontsumorako direla, ekoiztutako animalien osasungarritasunarekin bat doa. Kezkarik garrantzitsuenetarikoa itsas elikagaien ekoizpenean ur ingurugiroan dauden kutsatzatzaileek ekoiztutako produktuetan duten efektua da. Kutsatzaileak gero eta kantitate haundiagoetan azaltzen dira eta gainera, gero eta kutsatzaile berri gehiago agertzen dira (Bevan et al., 2012; Roose et al., 2011). Kutsatzaileak ez dira bakarrik uretara izurtzen, animaliei emoten zaien elikagaiak ere kutsatuta egon daitezke (Dahle et al., 2010; Dobson et al., 2008; Sharma and Paradakar, 2010). Hoierariko kutsatzaile askok ere, animalien ongizatean efektu negatiboak izaten dituzte eta galera ekonomiko haundiak sortarazten dituzte. Gaur egun, kutsatzaileak antzemateko metodo ez-inbasiboen galera dago. Hare eta gehiago, kutsatzaile berriak detektatzeko metodorik ez dago, antzemate metodoak kutsatzaile bakoitzeko ezpezifikoki diseinatsen direlako eta gero eta kutsatzaile berri gehiago detektatzen direlako, adibidez gizakion kontsumorako uran (Dahle et al., 2010; Roose et al., 2011).

Tesi honek, monitorizazio metodologia ez inbasibo bat garatzen du non arrainak Alerta Sistema Biologikoa (Biological Warning System - BWS) giza erabiltzen dira. Arrainak, euren igeriketa jokabidea aztertuz, sensore bat balira kontzideratzen dira. Metodologia honen abantailarik esanguratsugarrienak dira: i) teknologia eskuragarria bideratzea, ii) monitorizazio on-line-a uzten duela eta iii) mota ezberdinetako kutsatzaileekin, ezagun ala ezezagun, lan egiteko ahalmena izango duela.

Azkenik, proposatutako Alerta Sistema Biologikoa beste hierarkia handiagoko sistemekin egon beharko luke harremanetan eta sistema guzti hauen emaitzak ekoiztutako produktuen trazabilitate agiriekin batera joan beharko lukete azken produktuak merkatuara heltzerakoan. Hierarkia handiagoko sistema hauek Arrizku Analisis eta Punto Kritikoen Kontrol (Hazard Analysis and Critical Control Point - HACCP) eta Arrain Ongizate Segurtasun Sistemak (Fish Welfare Assurance System - FWAS) dira hain zuzen ere (van de Vis et al., 2012).

Hipotesia

Tesi hau ondoko hipotesian oinarrituta dago:

Arrain multzo batek sentsore biologiko bat izango balitz bezala jokatuko du; arrainen jokabidea, arrainen erantzun biologiko eta fisiologikoak batzen dituena, kanpo estimuluen aurrean modu ez inbasiboan neurtu daitekelako. Honek, arrain-sistemaren jokabidearen aldaketa on-line monitorizaziorako tresna gisa erabiltzea bideratzen du.

Helburuak eta ekarpenak

Tesi honen helburu garrantzitsuena arrainak sentsore giza erabiliz, arrainen ongizatea on-line monitorizatzeko eta akuikultura industrian aplikatzeko tresna baten disenua eta eraikuntza da. Honetarako, jakintza alor anitzak jorratzen dira, hala nola biologia, etologia, ingurugiroaren ikuskaketa, arrainen ongizatea, elikagain kalitatearen fidagarritasuna, sistemen ingenieritza edota seinaleen prozesamendu ez-lineala.

Lupia (*Dicentrarchus labrax*) izan da Tesi hau garatzeko aukeratutako arrain mota bi arrazoi nagusirengatik. Batetik, mediterraneo itsasoko herrialdeetako akuikultura produkzioan oso hedatuta dagoelako eta, aurrekoarekin loturik, lupiaren produkzioan arrainen ongizatearen eta azken produktuaren kalitatea oso garrantzitsua delako, gehienbat gizakion kontsumora bideraturiko produktua delako hain zuzen.

Bigarren mailako helburuak, proposatutako tresnaren ebaluazioarekin lotuta egoteaz gain, diseinatutako sistemak aldagai ezberdinen menpean duen portaera aztertzen dute, hala nola:

- Arrain-sistemaren erantzuna arrain kopurua aldatuz.
- Arrain-sistemaren erantzuna arrainen dietari selenioa gehitzerakoan.
- Arrain-sistemaren erantzuna urari kutsatzaile neurotoxikoa den metilmekurioa gehitzerakoan.

Hirugarren eta azken helburua, aurreko bi helburuen bitartez garatutako informazioa, ezagutza-eredu batetan isladatzearekin dator bat.

Helburu hauekin loturik, Tesi honek ondoko ekarpenak ditu:

- Sistemen ingenieritzan oinarritutako lan metodologia berri bat garatu da.
- Ez-inbasiboa, moldakorra, merkea eta Alerta Sistema Biologiko sistema batetan egokitzeko gai den irudi analisian oinarritutako tresna bat garatu da.
- Perturbazio bati erantzunez arrain multzoaren mugimendu ereduan oinarrituriko sentsore bat garatu da.
- Arrainen multzoaren igeriketa ereduari aplikatu ahal zaizkion zenbait seinale prozesaketa metodo ez-lineala garatu dira.

- Arrainen erantzuna kanpotiko perturbazio baten aurrean modelatzen duen eredu matematikoa garatu da.
- Garatutako metodologia eta monitorizazio tresna zenbait kasu experimentaletan aztertu da.

Ikerketa Metodologia

Ikerketa metodologiaren aldetik, Glass-ek (1995) proposatutako pausu berdinak jarraitu dira. Honela, Tesia lau faseetan banaturik dago: jakinarazte fasea, fase proposizionala, fase analitikoa eta ebaluaketa fasea. Era berean, erabilitako metodologia zati zientifiko eta zati enpiriko baten batura da. Ikerketa metodologia honek erdiesten dituen onurak ondokoak dira:

- Erabilitako ikerketa metodologia arrainen multzoak eta bere ingurugiroak osatzen duten sistemaren dinamika ulertzeko ezin bestekoa izan da.
- Tesian garatuta tresna zenbait kasu partikularretan aztertu da. Honela, tresnaren onurak ez ezik bere mugak ere aztertu ahal izan dira.
- Garatutako tresna benetako akuikultura instalazio batetan inplementatzeko bideragarritasuna aztertu da Norbegiako “Centre for Autonomous Marine Operations and Systems (AMOS)” Ikerketa Zentruan hiru hilabeteko ikerketa egonaldiari esker. Egonaldia Europa Batasuneko ikerlari mugikortasun beka batekin finantziatu da —European Economic Area (EEA) Researcher Mobility and Co-operation Grant, NILS Science and Sustainability Programme.
- Publikatutako lanen maila, kalitatea eta irismena handiagotu da.
- Etorkizunean, ikerketa proiektu eta gertaeretan parte hartzeko ahalmenaren handiagotzea, ikerlariaren kontaktu sarearen handiagotzearekin batera.

Azkenik, Tesiaren zati experimentalak Yin-en (1993) kasu-ikerketaren oinarriak jarraitzen ditu. Kasu-ikerketa metodoa ezagutza induktiboa sortzeko erabiltzen da eta eguneroko gertakari erreal eta komplexuei aplikatzerakoan baliagarritasun handia erakusten du.

Lanaren Garapena

Tesi honen lehenengo zatian, irudi analisian oinarritutako metodologia ez-inbasibo bat garatzen da. Metedologia hau, video grabaketa, irudi prozesaketa eta seinale ez-linealen analisi eta prozesaketan datza. Arrain multzoaren erantzuna gertaera estokastiko edo aleatorio baten aurrean aztertzen eta neurtzen da. Irudietan agertzen diren objetuen antzematea, fluxu optikoko algoritmo baten bitartez burutzen da. Honela arrainak detektatu ez ezik, irudien atzealdea eta bestelako efektu ez desiratuak ezabatzen dira. Azkenik, arrain multzoaren zentruak jarraitzen duen ibilbidea, Shannon (Shannon, 1951, 1948) eta permutazio entropia (Bandt and Pompe,

2002) batetik; eta Higuchi (1988), Katz (1988) eta Castiglioni-k (2010) proposatutako Katz algoritmoaren aldaketaren bitartezko dimentsio fraktal algoritmoez aztertzen da.

Garatutako monitorizazio tresna hiru kasu partikularretan aztertu zen. Lehenengoan, hiru lupia multzo aztertu ziren. Horietariko bi antzekoak ziren (kontrolak eta elastomero batekin markatutakoak), eta hirugarrena aldiz, 9 egunez metilmerkuriodun (4 µg MeHg/L) uretan murgildutako arrainez osoturik zegoen. Shannon-en entropia eta Katz-Castiglioni-ren algoritmoek izan ziren emaitza onenak erakutzi zutenak. Esan daiteke biek, arrainen erantzunak modu ez inbasibo batean kuantifikatzeko garaian, ahalmen nahikoa erakutsi zutela. Hala ere, Katz-Castiglioni-ren algoritmoak Shannon-en entropia baino askoz ere pisu konputazional handiagoa erakutsi zuen.

Hasiera baten, arrain multzoa osotzen zuten animalien kopurua inolako eraginik euki zitzakela animalia sistema osoaren jokabidean garatutako aztertze metodologiari dagokionez, ez zen uste. Honetan sakontzeko, bigarren kasu partikular bat diseinatu zen, non sistemako aldagai bakarra arrain kopurua zen. Bigarren kasu partikular honen helbururik garrantzitsuena sistemarentzako Lan Puntuaren zehazpena zen, hau da, arrainek inolako perturbazio barik daudeneko egoera dinamikoa. Monitorizazio tresna doitzeko eta perturbazioak egoera "normaletik" bereiztu ahal izateko, Lan Puntuaren zehazpena ezin bestekoa da. Honetarako bi experimentu disenatu ziren:

i. Arrain kopuruaren murrizketa. Arrain kopurua 50etik 1era pasatu zen 4 asteren buruan (50, 25, 13 eta arrain 1 aste bakoitzeko).

ii. Arrain kopururaren hazkuntza. Sistema osotzen duten arrainen kopurua 1etik 5era hazi zen egunero arrain berri bat sartuz (1, 2, 3, 4 eta 5 arrain egun bakoitzeko).

Hirugarren kasu partikularrean, aldiz, arrainen urari bi sustantzia ezberdin gehitu zitzaizkion. Batetik, sodio selenitoa (Na_2SeO_3, 10 µg/L) gehitu zitzaion 7 egunen zehar eta bestetik metilmerkurioa (4 µg MeHg/L) 14 egunen zehar.

Tesiaren azken atalean, aurreko kasuetan garatutako jakituriarekin ezagutza eredu bat eraiki da. Eredua, 3 azpi-ereduez osatuta dago, zeintzuk sistemaren eguneroko erantzuna eta Shannon-en entropiaren emaitzak, egoera basalean, gertaera aleatorio bateri erantzunez eta azken bien arteko erlazioari begira, integratzen dituzte.

Ondorioak

Ondoren Tesiaren ondoriorik esanguratsuenak adierazten dira:

1. Arrainen monitorizaziorako tresna garatu da. Tresnaren oinarrian irudi eskuraketa eta prozesaketa egoteaz gain, arrain multzoak osatzen duen zentruaren ibilbidearen analizi ez-lineala ere badago.
2. Arrainen sistemaren dinamika aztertzeko garaian Shannon-en entropia parametrorik onena izan da.
3. 1 eta 5 arrainen artean, lubia arrain sistemaren Shannon-en entropia eta sistema osotzen duten arrain kopuruaren erlazioa oso estua da.
4. Lubia arrain sistemaren eta Shannon-en entropiaren arteko erlazioa exponentziala da.
5. Garatutako tresna kasu partikularrei aplikatzerakoan era egokian lan egin du. Honek, ikerketa lanaren hasierako Tesia berretsi du.
6. Espero zen bezala eta garatutako tresnak konfirmatuta, urari gehitutako sodio selenito kontsentrazioak ez du inolako efekturik izan lubia sistemaren Shannon-en entropian.
7. Era berean, garatutako tresnak konfirmatu du urari gehituriko metilmerkurioak efektu ezezkorra izan duela lubia sistemaren Shannon-en entropian.
8. Tresnaren aplikazioak kasu partikularretan lubia sistemaren Shannon-en entropiaren eguneroko balioa ez ezik bere egunean-eguneko bilakaera kontutan hartu behar dela erakutsi du.
9. Garatutako ezagutza eredua lubia arrain sistemaren jokabide erantsunaren eguneroko bilakaeran oinarrituriko 3 azpi-ereduz osoturik dago: Azpi-eredu basala, gertaera aleatorioaren erantzuneko azpi eredua eta aurreko biak erlazionatzen dituen azpi-eredua.
10. Garatutako tresna, zenbait hobekuntzekin, on-line monitorizazio arkitektura batetan inplementatzeko gaitasuna duela esan daiteke. Eta beraz, era basatian nahiz akuikulturan, arrain multzoak monitorizatzeko edo/eta ur kutsadura antzemateko ingurumen-programetan tresna baliagarria izan daiteke.

Beraz, ikerketa lan honen Tesia arrainak sentsore biologiko moduan erabil daitezkela da; kanpo perturbazioen eraginez euren portaeran agertzen diren aldaketak modu ez-inbasiboan neurtu daitezkelako.

Gerorako Lana

Gaur egun Errezirkulazio Akuikultura Sistemek (Recirculating Aquaculture Systems - RAS) gero eta aplikazio handiagoa dute munduan zehar. Tesi honetan oinarrituriko teknologia baten garapenak zekulako erabilgarritasuna eukiko luke mota honetako sistemetan, batez ere, produkzio unitate osoa monitorizatu daitekelako. Era berean, ur kutsadura antzemateko ingurumen-programetan tresna baliagarria izan daiteke. Azken honetarako, teknologia honetan oinarrituriko ikusketa guneak garatu beharko lirateke.

Urpean lan egiteak desabantail ugari ditu. Hau ekiditeko datu eskuraketa teknika ezberdinak aztertu beharko lirateke, hala nola izpi-infragorriak, sonar teknologia edota irudi hiperespektralak zenbait esatearren.

Azkenik, teknologia honetatik lortutako datuak bestelako tekniken bidez lorturiko datuekin bat egin beharko lirateke. Honela, arrainen datu biokimiko, histologiko eta fisiologikoez gain, teknika ez-inbasiboen bidez neurtutako igeriketa jokabideak ere kontutan hartu beharreko parametroa izan beharko luke.

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