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New Development in Aflatoxin Research: From Aquafeed to Marine Cells

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1. Introduction

Available data on the real impact of aflatoxins on farm aquatic species are very limited. Since long time, aflatoxin B1 (AFB_1) has been considered the most potent food-born hepatotoxicant, frequently found in animal feedstuff. At present, it has been reported as responsible agent in unforeseen outbreaks of fish mortality due to acute or chronic aflatoxicosis, mainly well documented in freshwater species. The lack of information on the incidence of aflatoxicosis in marine reared teleosts may be partially due to the difficulty in accurately diagnosing the disease in fish, as well as to the lack of specie-specific *in vitro* models for toxicity studies.
In this work: 1) we have verified that pelletted fish feed might be considered as sources of AFB_1 contamination in aquaculture due to the isolation and identification of blue eye fungi (*Aspergillus* spp., *Penicillium* spp.) in feed samples, as well as other several genera (*Fusarium*, *Cladosporium*, *Alternaria*, *Geotrichum*, *Mucor*, *Rizophus*, *Acremonium*); 2) we have performed an *in vitro* evaluation of AFB_1 potential cytotoxic on *Sparus aurata* hepatocyte primary cultures (SaHePs), using a multiple endpoint screening. Our results demonstrate that seabream hepatocytes are highly sensitive to AFB_1 exposure and especially indicate three distinct pathways of cytotoxic response: necrotic cell death, apoptotic cell death and uncontrolled cell proliferation; 3) we have compared the dose response curves obtained by measuring the bioluminescence of *Vibrio fischeri* upon AFB_1 exposure to those obtained from *in vitro* cell culture system. Results show equivalent and overlapping toxic responses with those from seabream hepatocytes.

2. Impact of Aflatoxins in aquatic species

Aflatoxins are the most potent natural toxic metabolites produced by toxinogenic strains belonging to molds of the *Aspergillus* genus contaminating foods, feed ingredients and products of animal origin. Since their discover in the '60s, the aflatoxins poisoning continue to represent a growing potential threat to human and animal health based on their carcinogenic, immunosuppressive and other severe adverse effects (Chavez-Sanchez et al. 1994; Halver 1969; Han et al., 2010; Jantrarotai & Lovell 1990; Sahoo et al., 1996; Santacroce et al., 2008). The real impact of aflatoxins on farm animals have been largely studied especially in mammalians or in

the zootechnical field of terrestrian vertebrates. In contrast, very limited data on aquatic species are now available (Han et al., 2008; Lovell, 1992; Murjani, 2003; Pestka, 2007). Among all aflatoxins, aflatoxin B1 (AFB_1) is considered the most potent food-born hepatotoxicant frequently found in animal feedstuffs and responsible agent in unforeseen outbreaks of fish mortality attributed to aflatoxicosis, well documented in freshwater species since long time (Agag 2004; Cagauan et al., 2004; Santacroce et al., 2008). Aquatic species have shown dissimilar susceptibility to the hepatotoxic and carcinogenic effects of AFB_1 depending on the particular species . In fact, the fish susceptibility to AFB_1, largely studied for more than 50 years in USA and North Europe in freshwater fish and crustaceans, seems to be related with interspecies variations of AFB_1 biotransformation efficiency (Eaton & Groopman, 1994; Hendricks, 1994; Wales 1970). The increased use of plant origin ingredients in aquafeed formulations has intensified the potential onset for aflatoxicosis in fish farming systems due to the carryover of high loads of aflatoxin contamination by vegetable sources (Cagauan et al., 2004; Ellis et al., 2000; Fegan, 2005; Naylor et al., 2009; Spring, 2005). Aflatoxin production by the most toxinogenic strains can occur directly in the field, during insiling, feed formula preparation, and also during improper feed storage in the farm. On the other hand, the thermal treatments, applying high temperature pelleting procedures, even though destroy the mould but do not inactivate the heat-stable toxins present in spores and mycelium. Toxins accumulate in fish meal thus representing an high risk for the farmed species and then for the customer health and safety (IARC, 1993). As a result, the problem of aflatoxin contamination in aquaculture has amplified. Several studies revealed that AFB_1 residues can be retained in aquatic animal tissues, giving rise to potential public health risks after ingestion (Han et al., 2010; Messonnier et al., 2007; Puschner 2002; Tacon & Metian, 2008). Moreover, the presence of aflatoxins decrease the nutritional value of administrated feed in fish farm, both affecting the fish welfare status and the product quality (Hassan et al., 2010; Naylor et al., 2009). In intensive aquaculture, the features of administrated feed play a main rule being the major alimentary source involved with the fish growing and their nutritional requirements. The cases of acute intoxication by aflatoxin are almost rare and exceptional, while the chronic toxicity is the serious and most prevalent problem, because of AFB1 carcinogenicity upon long term microexposures. When moderate to high doses of aflatoxin are ingested, fish develop an acute intoxication, called acute aflatoxicosis, that generally gives rise to poor heath and fertility, loss in productivity, reduced weight gain, and immunosuppression (Stewart & Larson, 2002). Chronic aflatoxicosis occurs when low to moderate doses of aflatoxins are ingested over a long period of time. Generally, it is difficult to recognise or diagnose this condition because of its slow, subclinical trend. The majority of clinical signs is related to chronic status, such as impaired liver function, reduced feed efficiency, weight loss, increased susceptibility to secondary infectious diseases, necrosis and tumour development in liver and other organs, and increased mortality (Murjani, 2003). More insidious, pathological signs occur as a consequence of prolonged dietary exposure, causing genotoxic, tumorigenic and teratogenic, hormonal or neurotoxic effects in fish, as well as in humans. Chronic aflatoxicosis is of great concern in aquaculture systems, since it was found to be implicated both with a gradual decline of reared fish health status and with decreased stock quality. While considerable epidemiological data have been obtained on AFB_1 adverse effects in humans, farm animals and freshwater species, there is a substantial need to obtain such data especially on aquacultured euryaline fish (Santacroce et al., 2008).

The effect of AFB_1 on marine teleosts is quite unknown, although AFB_1 feed contamination is becoming of increasing interests in marine aquaculture (El-Sayed & Khalil, 2009). Even though

the problem of aflatoxicosis in fish was discovered about 50 years ago, sudden outbreaks of fish mortality continue to be reported, suggesting that the problem is still misunderstood and that scarce preventive measures have been adopted (Santacroce et al., 2008). At present, there is a gap in information regarding differences in AFB_1 susceptibilities in marine-reared fish. This means that the real exposure risk to AFB_1 in such species is still not understood.

Objectives of this work were: 1) to verify that pelletted fish feed might be considered as sources of AFB_1 contamination in aquaculture, isolating and identifying the contaminating toxigenic moulds; 2) to perform an *in vitro* evaluation of AFB_1 cytotoxic potential on *S. aurata* hepatocyte primary cultures, using a multiple endpoint screening; 3) to compare the dose response curves obtained by measuring the bioluminescence of *V. fischeri* upon AFB_1 exposure to those obtained from *in vitro* cell culture system.

3. Case report in aquaculture: Contaminated feeds

Most toxigenic molds, able to produce toxins, belong to the *Aspergillus*, *Penicillium*, and *Fusarium* genera (Moss, 1998). Fungal life consists of two different steps, mold growth and mycotoxin production, each one requiring specific and restricted conditions. Secondary metabolites are produced by toxigenic molds at the end of the active growth and under favorable conditions; they can be collected both in spores and vegetative mycelium or secreted into the growth substrate (Moss, 1991). Such metabolites promote the competitive fungal survival, but are not necessary for the essential metabolic functions of the fungus; they are commonly associated with the sporulation process and usually require strictly environmental conditions (Sekiguchi & Gaucher, 1977). Within the same fungal species, toxigenic strains can produce different quantity of mycotoxin and different types of toxic secondary metabolites, even if they show the same metabolic activity and speed of growth. The ubiquitus nature and biosynthetic heterogeneity of fungi hardly favors mycotoxin contamination of feedstuff (Dragoni et al., 2000). In animal feed colonized by toxigenic fungi are commonly present several mycotoxins, often found unchanged after feed processing (Jackson et al., 1996), because of their highly stable chemical structure. Studies carried on mycotoxin contamination of feed and food have led to the identification of over 100 toxigenic molds and at least 300 mycotoxins (Miller & Trenholm, 1994; Sharma & Salunkhe, 1991).

Herein, we highlight the existence of fish feed contamination in samples of spoiled grain pellets taken from a sea bream farm, entirely covered by moulds and spores (Fig. 1). After the grain was removed from the bin, green and blue eye moulds appeared both on pellets and surfaces. Feed portions of 50 g were transferred to sterile glass beacker and mixed with 450 mL of Sabouraud broth. After vortexing for 30 minutes, the suspension was opportunely diluted (1:10 and 1:100) and 100 µL of suspension and each dilution were streaked, in triplicate, into Sabouraud agar and incubated at 25-30 °C. All the suspect colony types were selected, streaked onto Sabouraud agar to obtain pure cultures and screened for morphological characterization (Fig. 2).

The fungal species most frequently identified in the group of the blue eye fungi belonging to the taxa of Ascomycota were *Aspergillus* spp. (Fig. 3) and *Penicillium* spp. (Fig. 4). Numerous other fungi were isolated, and the genera, *Mucor* (Fig. 5), *Cladosporium* (Fig. 6), *Fusarium* (Fig. 7), *Geotrichum* (Fig. 8), *Alternaria*, *Rhizopus*, *Acremonium* were predominant among the moulds found. Acco rding to several authors, the identification of wrong storage conditions since the presence of blue eye group fungi, certainly indicates that the grain has been improperly stored.

Fig. 1. Fish feed contamination: spoiled pellets from a sea bream farm, entirely covered by moulds and spores

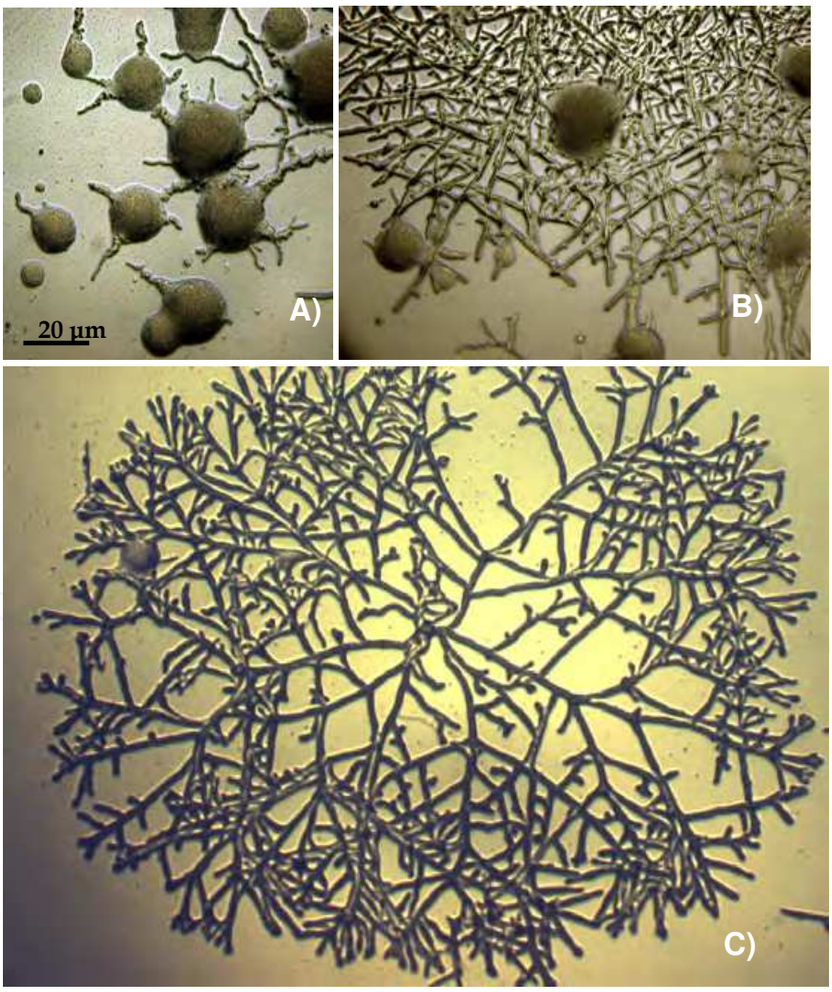

Fig. 2. Seed suspension on Sabouraud agar plate: A) Hyphal germination (x200); B) Hyphal elongation and branching (x200); C) Mycelium growth and mass of hyphae (x100)

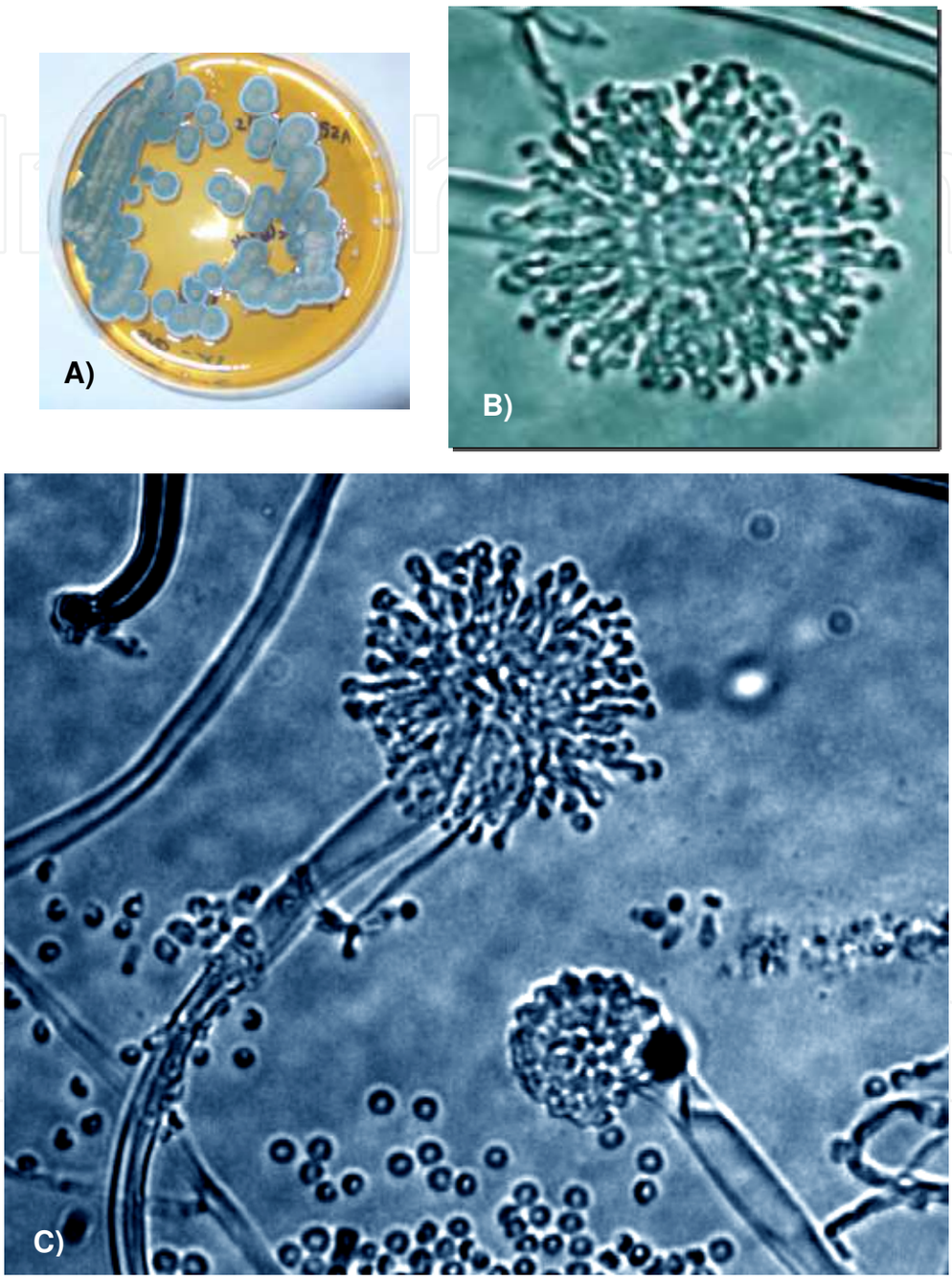

Fig. 3. *Aspergillus flavus* isolated from contaminated aquafeed administered to aquacultured seabream: A) mould growth on Sabouraud agar plate; B), C) Direct microscopy of a Scoth test on slide (x400)

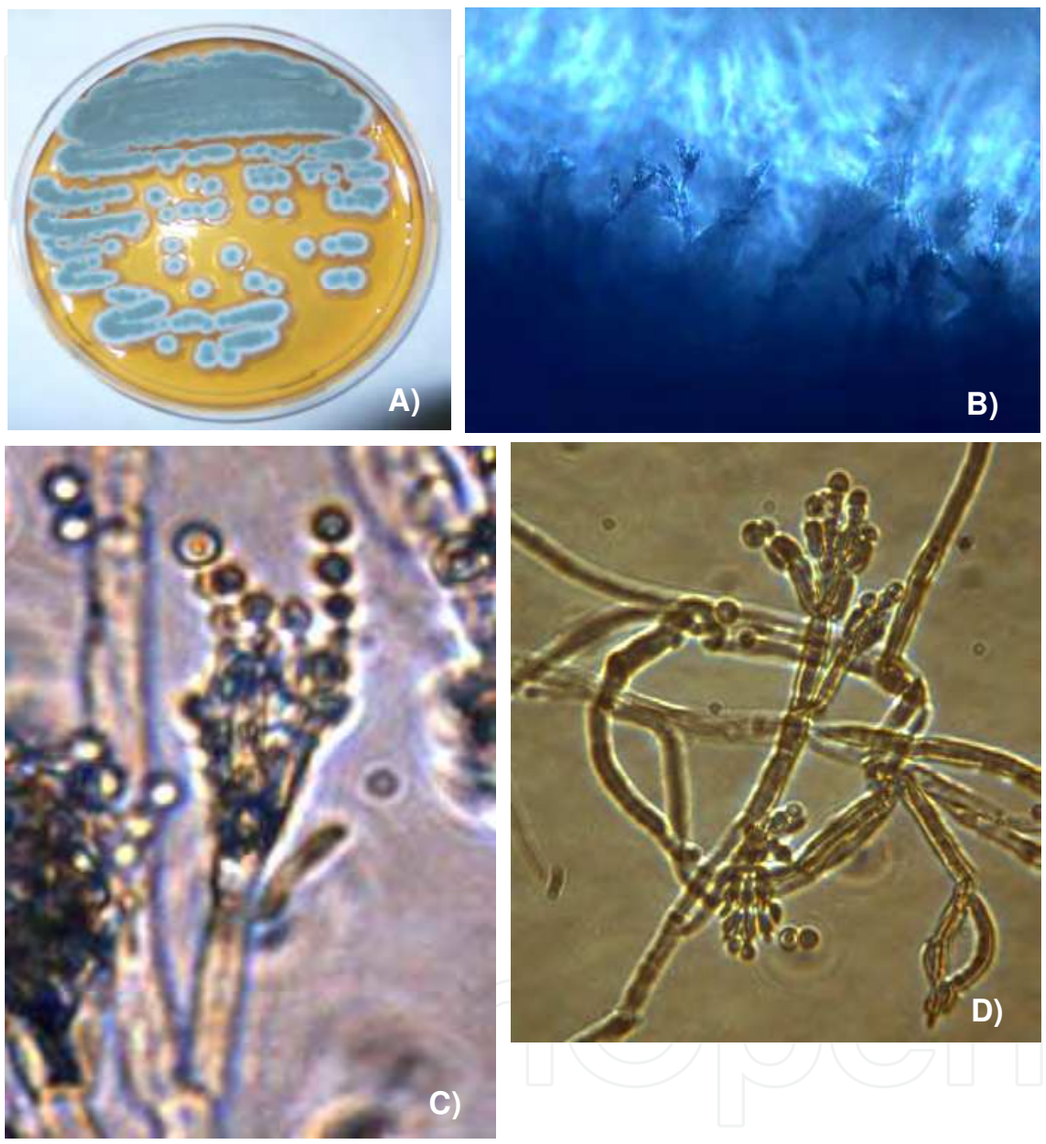

Fig. 4. *Penicillium crysogenum* isolated from contaminated aquafeed administered to aquacultured seabream: A) mould growth on Sabouraud agar plate; B) growth of aerial hyphae (x400); C) Conidial head two-stage branched (x600); D) Direct microscopy of a Scoth test on slide (x400)

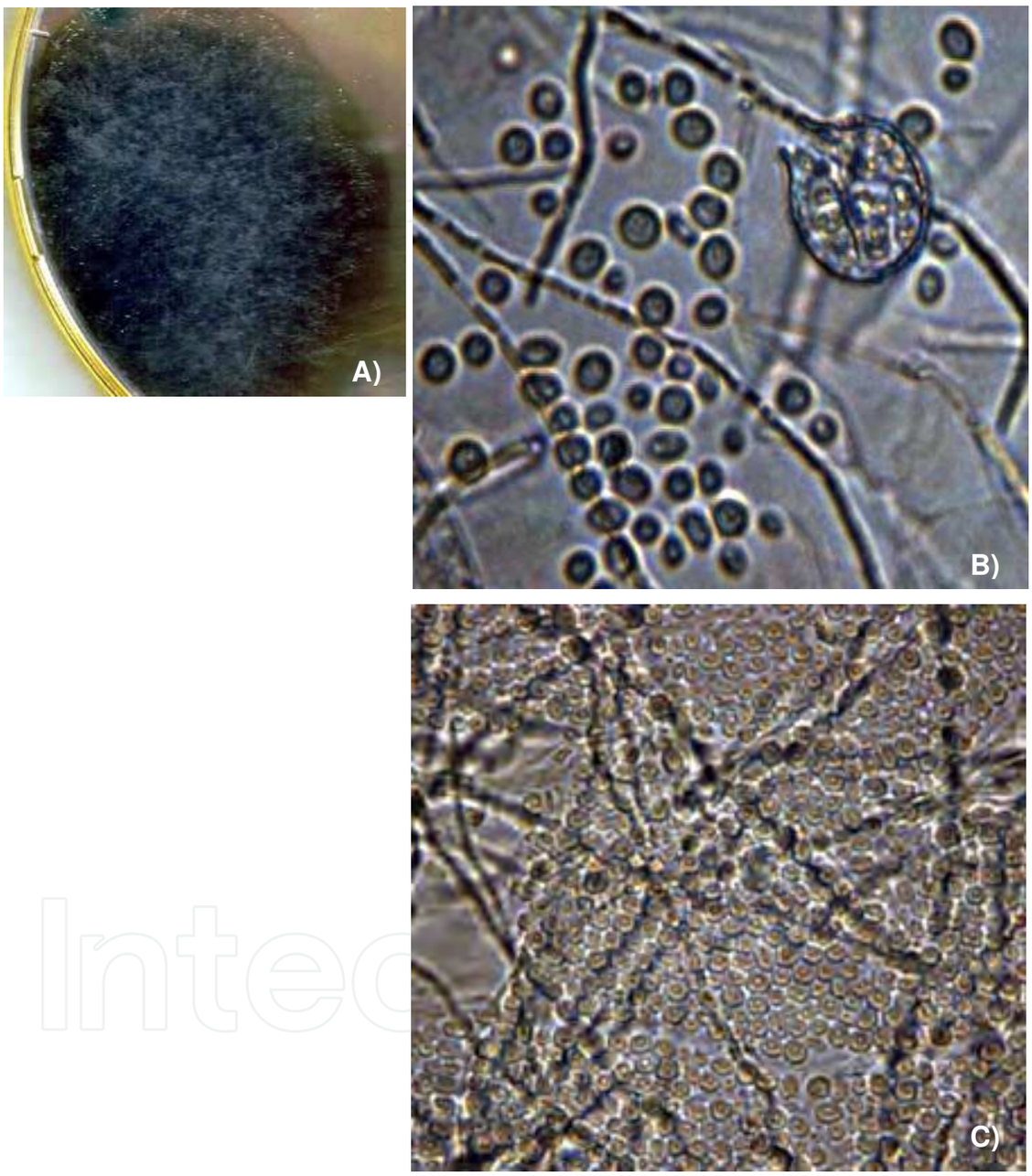

Fig. 5. *Mucor* spp. isolated from contaminated aquafeed administered to aquacultured seabream: A) mould growth on Sabouraud agar plate; B) dispersal of conidia from ascospores (x600); D) Direct microscopy of a Scoth test on slide (x400)

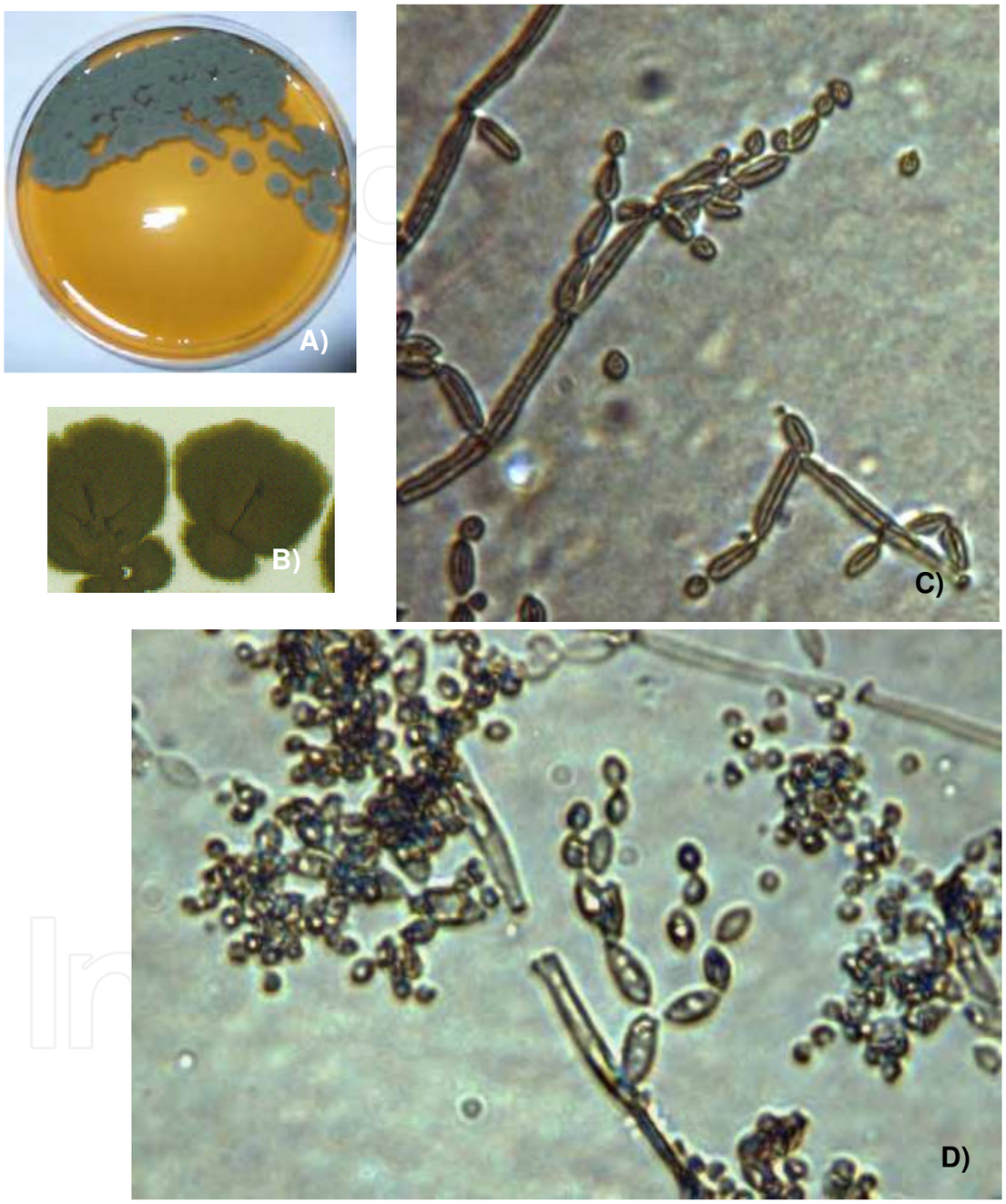

Fig. 6. *Cladoaporium* spp. isolated from contaminated aquafeed administered to aquacultured seabream: A) mould growth on Sabouraud agar plate; B) dispersal of conidia from ascospores (x400); D) Direct microscopy of a Scoth test on slide (x400)

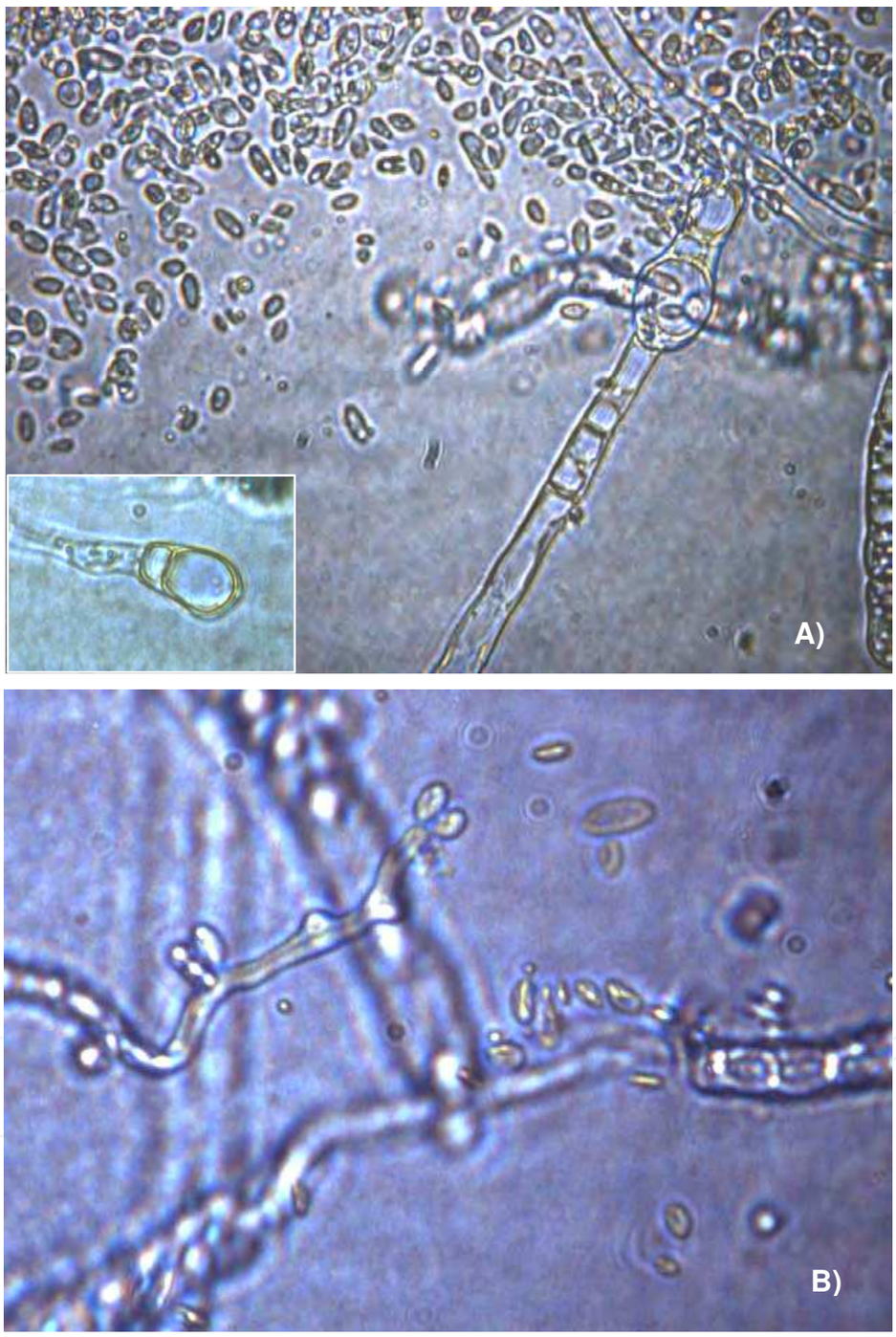

Fig. 7. *Fusarium* spp. isolated from contaminated aquafeed administered to aquacultured seabream: A) Direct microscopy: conidiophores with intercalary chlamydospores, and terminal clamydospore (left lower corner) (x600); D) Direct microscopy: conidiophores with terminal phialides and microconidial dispersal. (x600)

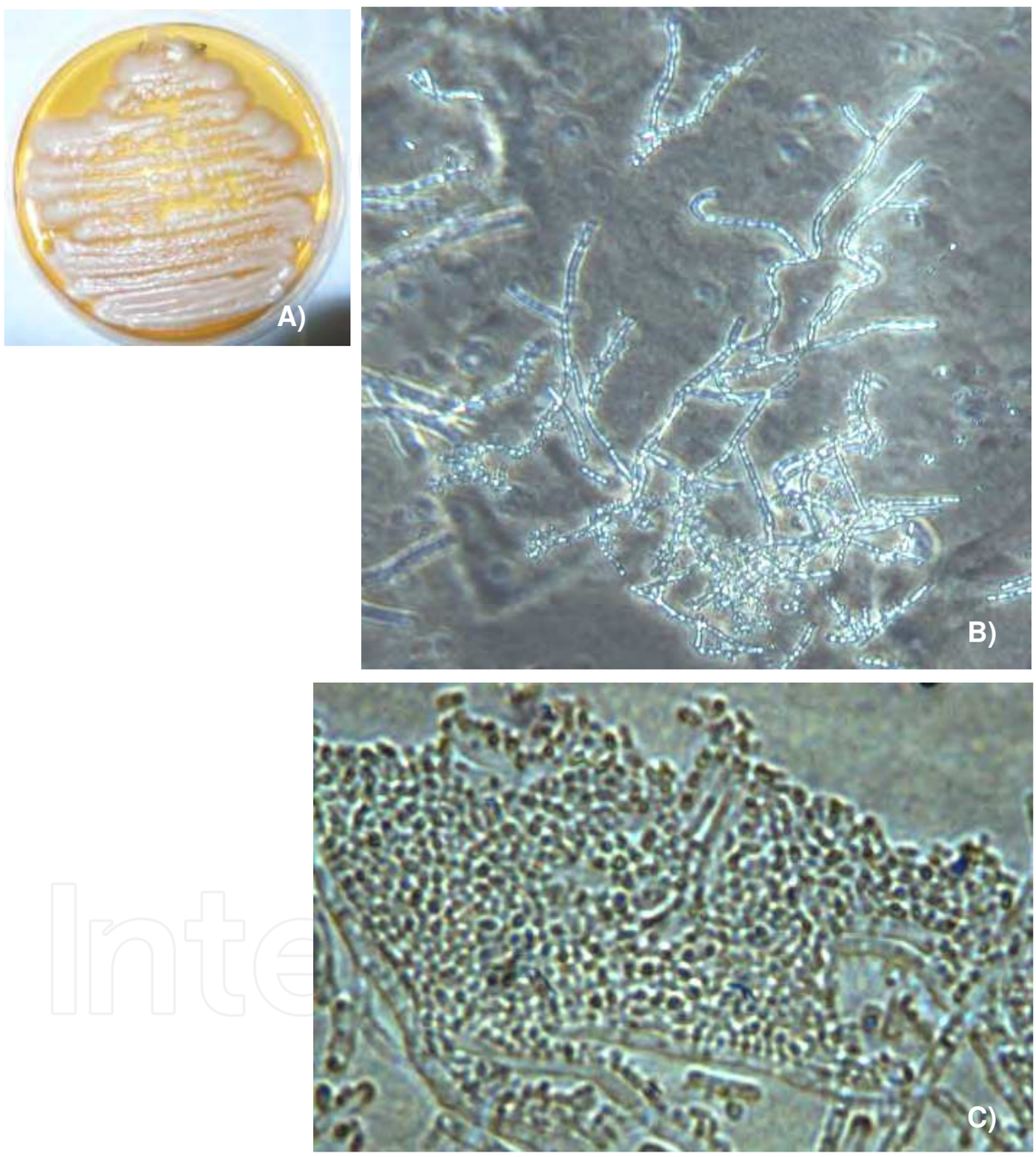

Fig. 8. *Geotrichum* sp. isolated from contaminated aquafeed administered to aquacultured seabream: A) mould growth on Sabouraud agar plate; B) (x200) fragmentation of undifferentiated hyphae; C) (x400). Direct microscopy: arthroconidia

4. New models of study: Primary cultures of marine teleostean hepatocytes

Based on the previous reported data, it may be assumed that the lack of information regarding the incidence of aflatoxicosis in marine reared species may be in part due to the difficulty in accurately diagnosing aflatoxicosis in fish, as well as to the lack of specie-specific *in vitro* models for toxicity studies. It is well known that animal cell cultures permit the comparison of species at a cellular level under equivalent conditions of toxicant exposure. Because of the insufficient availability of *in vitro* marine liver systems on a species-specific basis, comparison studies between AFB_1 cytotoxicity on freshwater and seawater fish cells have never been made. Although primary hepatocytes represent the most employed within all the *in vitro* liver models, an ideal hepatocyte system targeted for marine fish has not yet fully established. In order to overcome this issue, we have developed a new *in vitro* model which firstly describes the isolation and cultivation of hepatocytes from a marine Mediterranean teleost of great economic value, the gilt-head seabream (*Sparus aurata*), applying a method different from the ancient liver perfusion (Santacroce et al. 2010). In this method, seabream hepatocytes were quickly derived from the explanted liver, without any passage of liver perfusion *in vivo*, through several steps of mechanical separation, multiple enzymatic digestion and isopicnic cell purification. Previously works on isolation and cultivation of teleost hepatocytes were done following the two-step perfusion procedure, firstly described by Seglen (1976), followed by Mommsen et al. (1994) and then adopted from over forty years by other authors (Segner, 1998; Mommsen *et al., 199*4; Pesonen & Andersson, 1991). The protocol involved a first perfusion of the liver with a Ca_2^+-free balanced saline solution followed by a second perfusion with the digesting enzyme collagenase. The liver perfusion was the first important technique implemented for the preparation of primary hepatocytes (Guguen-Guillouzo, 1992). Firstly, the method was developed in rodents, (Berry & Friend, 1969; Seglen, 1976) and then improved to obtain hepatocytes from other animal sources like pig (Chen et al., 2002; Koebe & Schildberg, 1996), sheep (Clark & Vincent, 2000) and finally fish (Birnbaum et al., 1976; Blair et al. 1990; Braunbeck & Segner, 2000; Mommsen et al., 1994; Segner, 1998). Since the early 70s, this was the main method largely used for aquatic vertebrates, event though the main disadvantage of this procedure is the large size of animal (approximately 100 g/body weight) required to permit the *in vivo* abdomen insertion of perfusion devices connected to a peristaltic pump. Mitaka et al. (1995) proposed a selective separation method for obtaining and culturing small hepatocytes by using hyaluronic acid-attached carrier. This technique comprised the isolation of hepatocytes from the liver of adult rats by perfusion through portal vein according the method of Seglen (1976). Other alternative procedures established in freshwater fish involved cutting the liver into pieces and incubating in a solution of 0.5% of collagenase until full digestion (Mitaka & Kon, 2004). This procedure was outlined by Bouche et al. (1979) in carp but only after liver perfusion through the arteria coeliaca. In rare exceptions, alternative enzymes were used as single or mixture addition, but generally reported as components of the perfusion medium adopted during the liver perfusion (Braunbeck & Segner, 2000). Those used for the dispersion of fish hepatocytes were hyaluronydase, protease, elastase and nagarase (Mommsen et al., 1994) or trypsin, as reviewed by Braunbeck & Segner (2000). Only one study is reported in literature on the isolation of primary seabream hepatocytes, but still based on the liver perfusion technique according to Mommsen and colleagues (1994) (Bevelander et al., 2006). The liver was perfused via the heart for 15 min, then excised, cut in small pieces and incubated with a

solution of collagenase (0.3 mg/mL) for 45 min. Unfortunately, those hepatocytes showed only a limited survival, up to 1 week, suggesting a limitation in cell viability. The above method is not applicable with fish of relative small size, like juveniles or small aquarium fish. Hence, the pressing necessity to improve the isolation and tissue culture techniques involving cells for a better implementation of biotechnological methods on marine species.
The method herein described for isolating seabream hepatocytes differs from those previously cited for the medium composition, the type of enzyme mixture and concentration, digestion time and temperature, time and speed of centrifugation steps, filter type and selectivity, isopicnic separation of hepatocytes from nucleated erythrocytes, cultivation conditions (temperature, CO_2 tension, refrigerate incubator). *S. aurata* juveniles (30 ± 4 g mean body weight, n = 45) were used for establishing hepatocyte primary cultures (SaHePs) according to the new procedure, suitable for fish of small size (Santacroce et al., 2010). Hepatocytes were isolated as described previously by tissue physical disaggregation combined with enzyme digestion, and purificated by several steps of centrifugation (Santacroce et al., 2010). Freshly isolated hepatocytes were tested for viability by Trypan blue, counted and seeded at a density of 30,000 cells/cm² in 96-well plates Falcon BD previously pre-coated with collagen I. Cells were cultured in a refrigerate incubator at 18°C in humidified atmosphere of 97% air/3% CO_2 (Fig. 9).

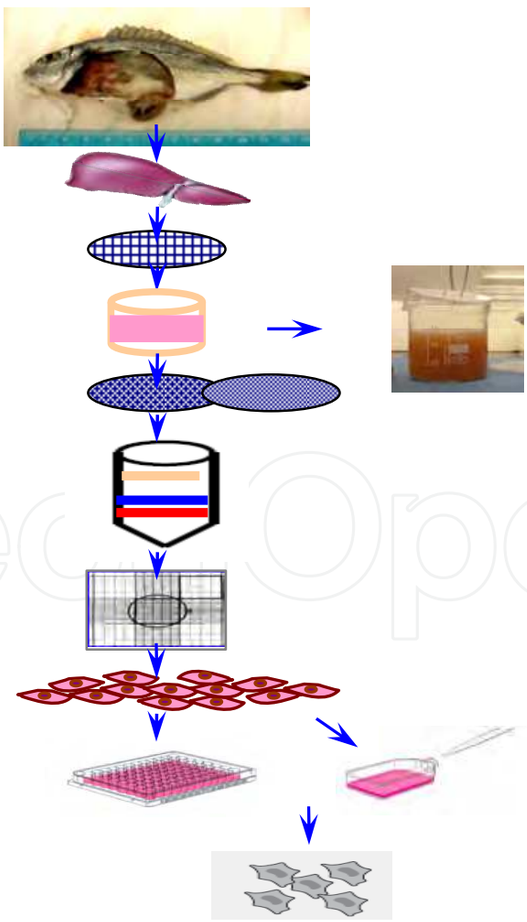

Fig. 9. Seabream hepatocyte primary culture protocol

The cells were grown in Leibovitz's L-15 medium with 2 mM L-glutamine, 10% FBS, 100 IU/mL penicilin, 100 µg/mL streptomycin, 100 µg/mL amphotericine, 50 µg/mL gentamycin, 1 mM Na Pyruvate, 5 mM D-Glu, 10 mM HEPES, 12 mM $NaHCO_3$, and supplemented with 20 mM NaCl, 0.05% ITS *plus* (insulin/transferrin/sodium selenite *plus* oleic acid/linoleic acid/BSA), 0.01 mM MEM non-essential amino acid, 0.01 mM MEM-vitamin mix, 0.1 mM ascorbic acid, and 0.01 µg/mL epidermal growth factor, 0.005 µg/mL hepatocyte growth factor. Cells were allowed to attach for 12 h, afterward fresh nutrient L-15 medium was added. After 24 h of incubation, the non-adherent cells were removed by washing wells twice with 1X PBS, then L-15 medium was changed every 24 h thereafter. Fig. 10 shows phase contrast images of *S. aurata* primary hepatocytes cultured on collagen I coated flasks during monolayer formation, development and differentiation. Starting from the third day to sixth, during the phase of monolayer development, SaHePs consisted basically of two cell types: small hepatocytes, represented by islands with tightly packed small cells, each containing multiple proliferating islets that grew inside, and large hepatocytes, formed by cords of large bright cells. Then, more multicellular islands appeared, and cords of large hepatocytes moved to fill the space between the islands of small hepatocytes. During development, most cells adopted morphological changes, showing a hepatocyte typical polygonal shape. In the second week from the seeding, monolayer reached about 70% of confluence. By the third week, cells formed a compact monolayer, fully differentiated. Morphologically, cells presented one or two nucleus with two large evident nucleolus, apparent cytoskeleton, and characteristic biliary canalicular structures in proximity of intercellular junction of two adjacent hepatocytes.

Fig. 11 shows immunofluorescence images of primary hepatocytes positive stained for several liver markers after 3 days seeding on to collagen I coated chamber slides: (A) Cytochrome P450, the major drug metabolizing enzyme located in the endoplasmatic reticulum; (B) Albumin, the most abundant protein synthesized by mature and functional hepatocytes; (C) CK-18, a special skeleton protein of hepatocyte; (D) production of extracellular matrix stained with Ab anti-CK-18-TRITC, (E) viable cells (green) and viable nuclei stained with DAPI (blue), (F) metabolic active cells in CFDA, carboxyfluorescein diacetate. G) and H) show the cell proliferative capacity: mitotic nuclei (white) are double immunolabelled with Ab anti PCNA, an intranuclear protein cell cycle dependent, which assists DNA polymerase delta during DNA replication, hence its expression is considered a marker of DNA synthesis, and PI, a nuclear counterstaining; resting nuclei are red, and viable cells are green (CFDA- FITC).

The whole microscopy analysis was performed, for brightfield and fluorescence, by a Motic AE31 Epi-Fluorescent Inverted Microscope, equipped with DAPI/TRITC/FITC fluorescence filter cube set. Digital image capture was performed by Moticam 3000C Cooled CCD digital color camera (3.3 Megapixel, 1/2'' CCD), capture system in origin Live Cam 1.0 (32-32) and Motic Images Advanced (V. 3.2) acquisition software (Motic, Seneco, Milan, Italy). Image analysis and assemblage was performed with Motic Images Advanced (V. 3.2) (Motic, Seneco, Milan, Italy) and Adobe Photoshop 8.0 (Adobe, Inc.).

5. AFB_1 exposure in two *in vitro* systems

The nature and the degree of possible harmful effects produced on living species by toxicants can be evaluated using both analytic laboratory tests and bioassays. The information obtained by these tests are useful for implementing environmental risk assessment. Although analytic chemistry is sensitive, the biological models offer more

information about chemical damage. In the last years, several biological models were used in both short- and long-term tests implementing various test organisms. Short-term tests are based on the assessment of quickly measurable parameters, such as bioluminescence in Microtox® system (Bulich, 1986), extensively applied. Long-term tests rate parameters such as cell viability or growth using as model human (Delmas et al., 2000; Gaubin et al., 2000), duckweed (Ince et al., 1999), or fish (Santacroce et al., 2010) cell lines.

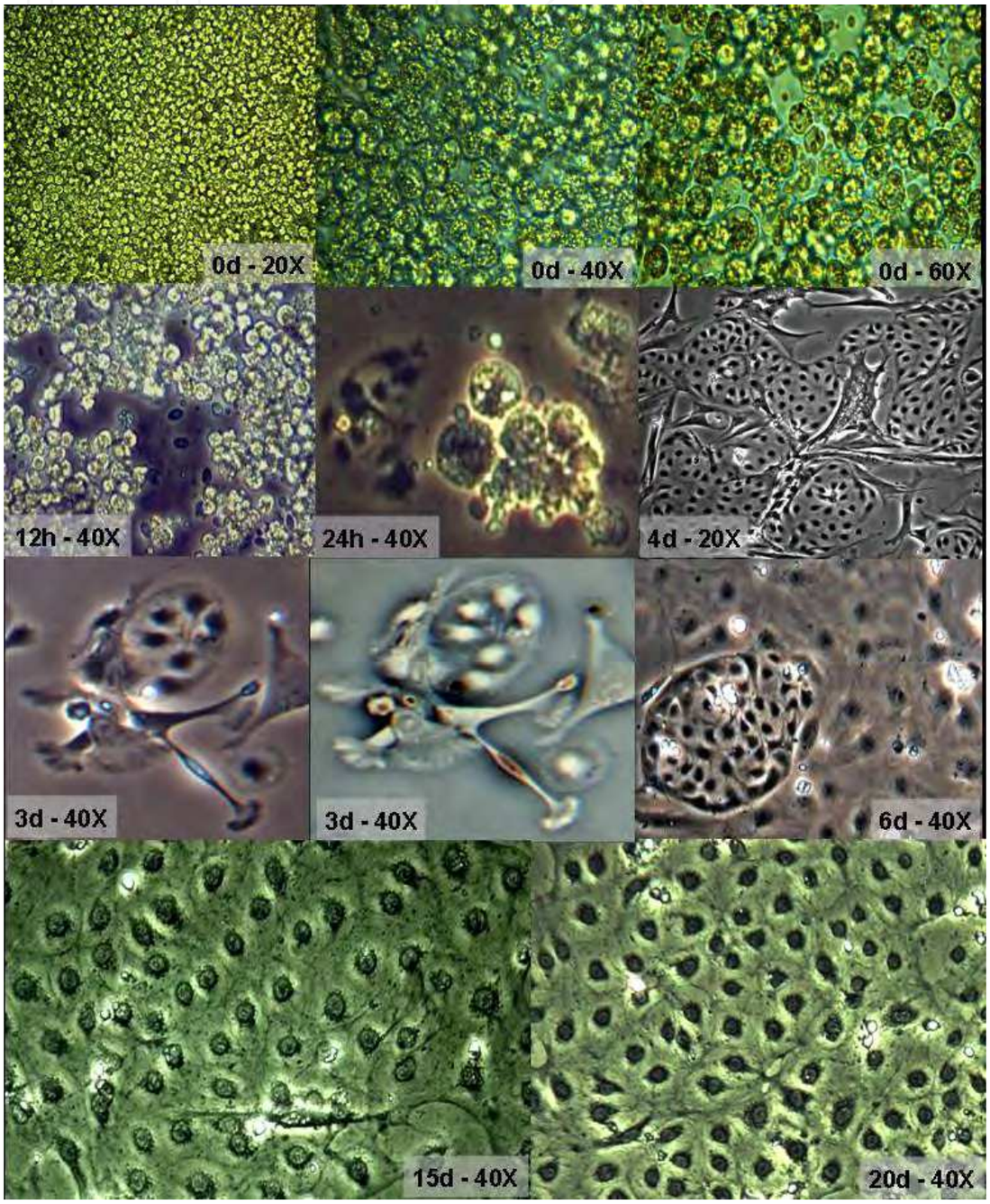

Fig. 10. *S. aurata* primary hepatocytes culture

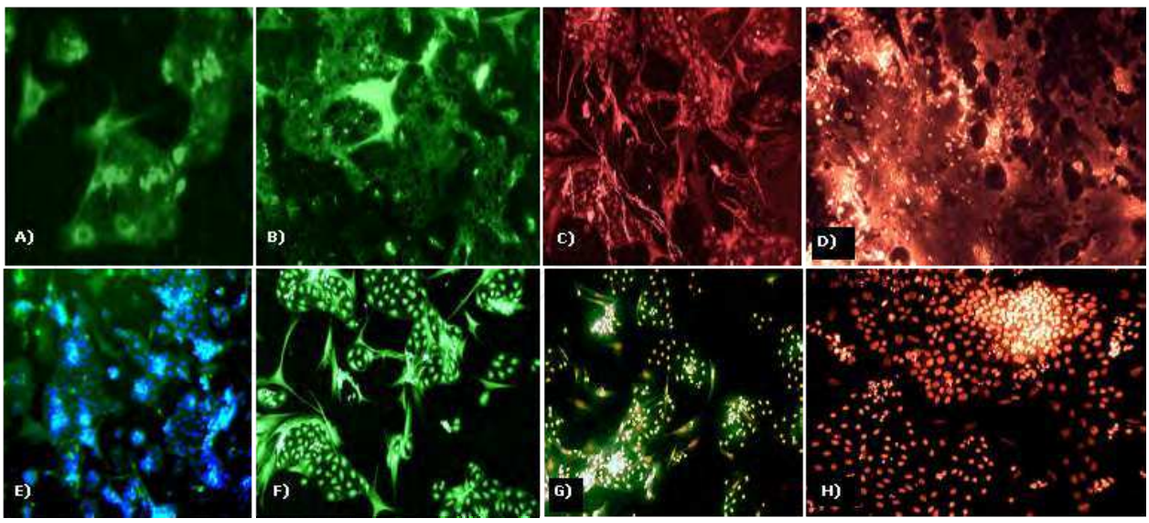

Fig. 11. Immunocytochemistry and morphology of seabream hepatocytes after 3 days seeding in Collagen I coated chamber slides. Immunofluorescence images show primary hepatocytes positive stained for several liver markers**:** (A) Cyp1A1/2-FITC, (B) Ab anti-Albumin-FITC, (C) Ab anti-CK-18-TRITC, (D) production of extracellular matrix stained with Ab anti-CK-18-TRITC; (E) viable cells (green) and viable nuclei counterstained with DAPI (blue) FITC/DAPI merged; (F) Metabolic active cells in CFDA (FITC); G) and H) show results pertaining to proliferative capacity: mitotic nuclei (white) are double immunolabelled with Ab anti PCNA-FITC and PI-TRITC, resting nuclei are red, and viable cells are green (CFDA-FITC), (G) CFDA-FITC/PI-TRITC merged; (H) Ab anti PCNA-FITC and PI-TRITC

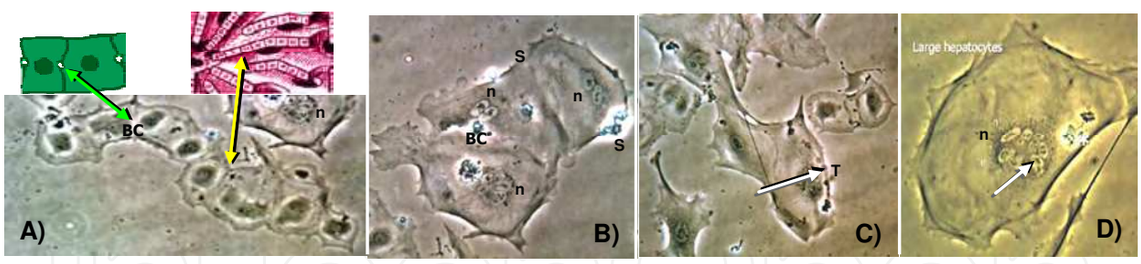

Fig. 12. Phase contrast images of primary primary seabream hepatocytes cultured at 18°C, 3% CO_2 on Collagen I coated T-25 flasks showing the apical, canalicular membrane with canaliculi resembling structures: a) cord of hepatocytes in culture; b) binucleated (n) hepatocytes with biliar canaliculi (BC) and granular secretions (S); c) transcytosis mechanism (T); d) mature polyploid hepatocyte highly differentiated

Based on the serious effects that aflatoxins can have on farm management and human health, the knowledge of their detection, toxicity, biosynthesis, and regulation is necessary to give proper responses to aflatoxin intoxication (Do & Choi, 2007).

5.1 AFB_1 effects on *Sparus aurata* hepatocytes

An *in vitro* evaluation of AFB_1 cytotoxic potential on SaHePs was carried out as second goal of this study. We performed a toxicity assessment by using a multiple endpoint screening. The

work was based on series of *in vitro* cytotoxicity and functional assays in order to: provide new information on the toxic properties of AFB_1 at cellular level, characterize the type and degree of damage, the threshold hazard dose for reared seabream, and the boundary between acute and chronic toxicity. SaHePs were treated with a wide range of AFB_1 concentrations (from 0.25 mg/mL to 0.001 pg/mL) for 24, 48 and 72 hours, thus mimicking acute and chronic conditions. After each exposure, hepatocytes were examined for morphologic alterations, viability and citotoxicity, and apoptosis induction. The cytotoxic activity of AFB_1 was characterized by measuring two different viability endpoints, such as the MTT assay, as marker of cellular metabolic activity, to check the mitochondrial dehydrogenase activity using 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) as substrate, and the neutral red (NR) retention assay to check lysosomal function upon AFB_1 exposure. Finally, the release of the cytoplasmatic enzyme lactate dehydrogenase (LDH) was performed as marker of lethality to check the membrane integrity. For each treatment time, the cytotoxic effect was determined as the half-maximal inhibiting concentration (IC_{50}) resulting in 50% of reduction in cell viability. The IC_{50} values were determined fitting data to a four-parameter logistic model by using a Hill function non-linear regression analysis with the GraphPad Prism v.5.00 software package. Apoptosis was evaluated by assessing the phosphatidylserine (PS) exposition in the outer leaflet of plasma membrane at the cell surface of dying apoptotic, using the Annexin V-Cy3.18 binding in fluorescence microscopy. This assay allowed to identify the subacute cytotoxicity by differentiating early apoptotic cells from viable or necrotic ones. Tumorigenesis was evaluated by the proliferating cell nuclear antigen (PCNA) labelling, an intranuclear protein cell cycle dependent considered as marker of DNA synthesis. Results showed that AFB_1 exhibited dose- and time-dependent cytotoxic effect, the IC_{50} being inversely related to the exposure time (Table 1). Dose-response curves obtained after 24, 48 and 72 h revealed that prolonged exposure times lead to a significant increase of the toxic potency of AFB_1 (Fig. 13).

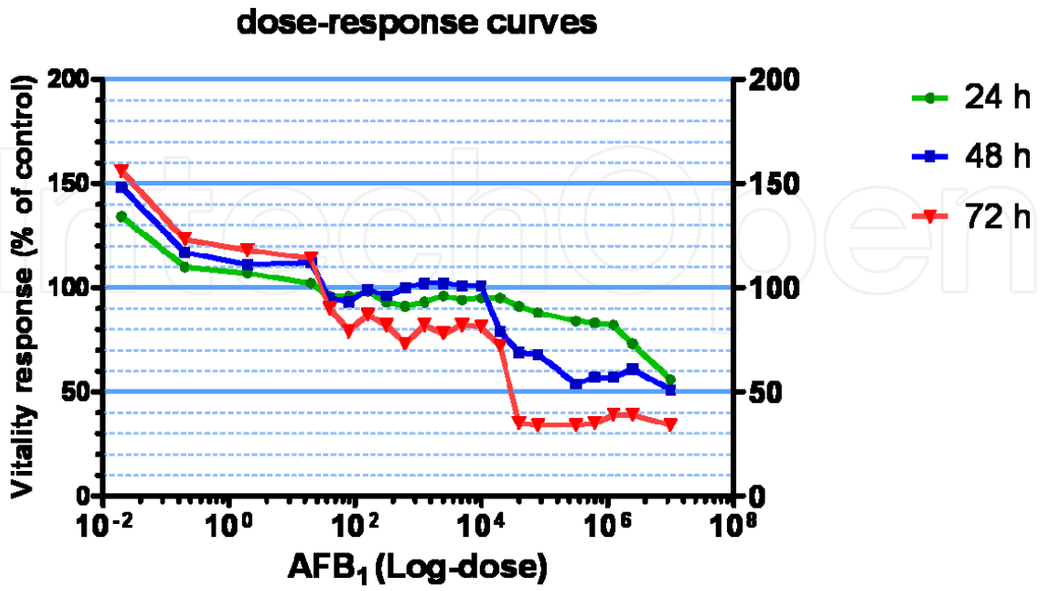

Fig. 13. Concentration-response curves for AFB_1 exposures on SaHePs

Although results showed that the viability endpoints used (NR, MTT) for measuring the AFB_1 cytotoxic potential were comparable, the IC_{50} value of the MTT was a more sensitive parameter of cytotoxicity (Table 1). In fact, the three cytotoxicity endpoints have been combined into a single value by applying the equation of Castano et al. (1994) which resulted strictly close to the MTT value, CI 0.067 µg/mL versus 0.609 µg/mL.

Assays	**IC_{50}-24h**	**IC_{50}-48h**	**IC_{50}-72h**
NR	3 µg/mL	0.3 µg/mL	0.03 µg/mL
MTT	5 µg/mL	0.6 µg/mL	0.06 µg/mL
LDH	4 µg/mL	3 µg/mL	2 µg/mL

Table 1. IC_{50} values at 24, 48, 72 h of NR, MTT and LDH assays

The maximum lethality response was assessed after 72 h exposure at 1.95 µg/mL (25% of metabolic activity), afterward cell death reached a plateau in almost 70-80 % of hepatocytes without any further damage recover up to 250 µg/mL. The dose-response curve at 72 h had a higher threshold but a steeper slope than 48 and 24 h. The release of LDH allowed to monitor primary necrosis over treatment times and to define the acute toxicity boundary. The threshold dose level (LOEC), where toxicity first appears, was estimated at 10 ng/mL, whereas the no-observable-adverse-effect-concentration (NOEC) was at 5 ng/mL. However, at doses within this apparent safe level, and approaching the baseline, cytotoxic effects and delayed secondary cell death were observed ranging from 0.02 µg/mL to 0.005 µg/mL, with signs of cell suffering. In this range cell vitality decreased in a time dependent manner, since about 0-5% of cells death was registered after 24 and 48 h, while at 72 h cell survival lowered up to a maximum of 20 % (Fig. 14).

In order to distinguish whether such increase in cell death after 72 h was due to primary necrosis or delayed secondary cell death, an immunocytochemical analysis was performed using direct immunofluorescence with the Annexin V-Cy3.18 staining. Apoptosis marker response confirmed that this apparent safe level hid a delayed mortality for apoptosis induction, even observable up to dose of 0.2 ng/mL. Figure 15 shows hepatocytes cultured in a 4-well slide (500 µL/well) exposed to the toxin for 24 h at doses of: 0 µg/mL (A) as normal control (annexin V-/6-CFDA+) with living cells marked in green; 1.9 µg/mL (B) (annexin V+/6-CFDA-) with necrotic cells in red; 0.02 µg/mL (C) (annexin V+, 6-CFDA+) apoptotic cells in orange; and 0.1 ng/mL (D) (annexin V+, 6-CFDA+) early apoptotic cells in green-yellow. At this latter dosage, cell membranes are still undamaged, even though cells are genotoxically compromised and designated to death by exposure time. Figure 15 (E) shows the typical apoptotic morphological changes upon exposure to 0.1 ng/mL for 72 h, characterized by detachment, swelling to dense rounded mass, cell shrinkage, apoptotic bodies cluster formation "popcorn like" and cell death with final lysis stage.

Below the non effect zone (0.005 µg/mL to 0.2 ng/mL), the response started to deviate up and down from the control baseline, identifying the hormesis zone. At lower doses, an increase in the occurrence of tumorigenic transformed cells was observed by PCNA labelling (Fig. 16 A, B). Presumably, the genotoxic DNA lesions, induced during the apoptotic pathway, may determine a reduced efficiency in the cell cycle check point, so contributing the development of transformed cellular phenotypes and tumours. In fact, the persistence of mutant cells, which evade the apoptosis, leaded to an increased number and dimension of tumoral foci over time as doses lowered. High proliferation was

registered after 72 h of exposure when AFB_1 was ranging from 0.2 pg to 0.001 pg/mL. This was confirmed by the number of mitotic cells positives for immunofluorescent labeling of DNA (Fig. 16 A, B, C).

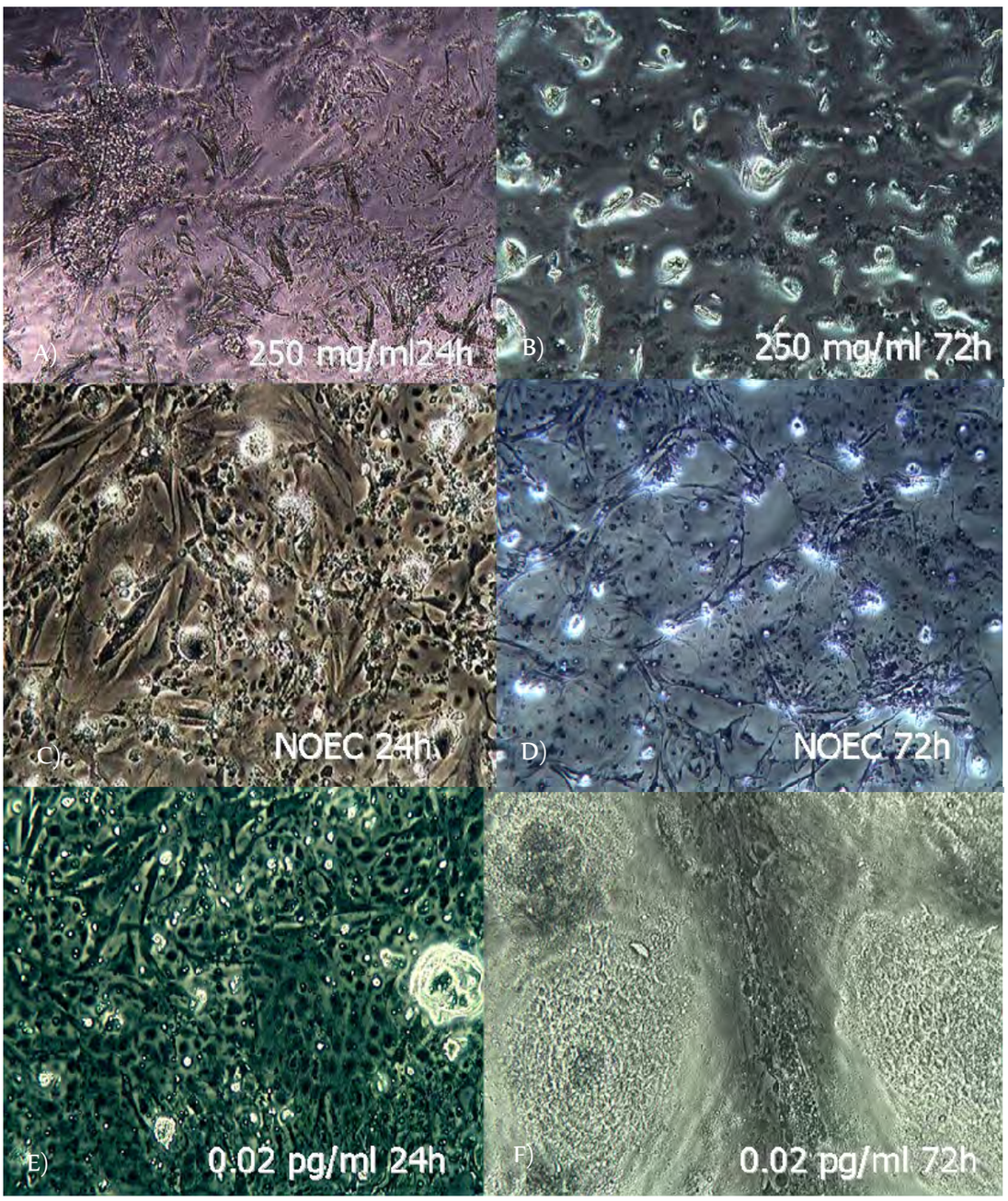

Fig. 14. AFB_1 damage on SaHePs after 24 h and 72 h of AFB_1 exposure (original magnification x200). A) and B) SaHePs exposed at 250 mg/mL; C) and D) SaHePs exposed at 5ng/mL; E) SaHePs exposed at 0.02 pg/mL after 24 h: cell shrinkage, pyknosis; F) SaHePs exposed at 0.02 pg/mL after 72 h: extensive cell proliferation with the loss of contact inhibition, and formation of multicellular overgrowth nodules (tumoral foci).

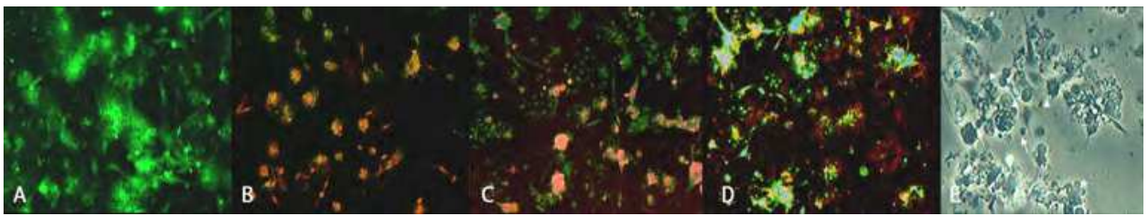

Fig. 15. Hepatocytes cultured in a 4-well slide exposed to AFB_1 for 24 h (original magnification x200)

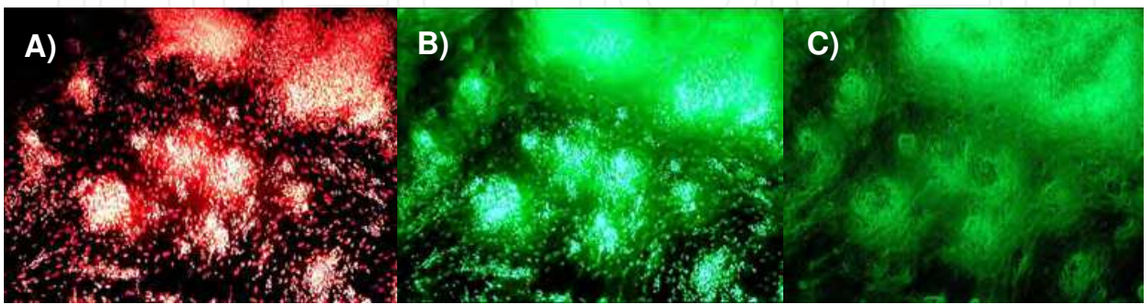

Fig. 16. SaHePs exposed to AFB_1 at dose of 0.001 pg/mL after 72 h. Tumoral foci formation: mitotic nuclei (white) are double immunolabelled with A) Ab anti PCNA-FITC and counterstained with PI-TRITC, resting nuclei are red; in B) and C) viable cells are green, B) PCNA-CFDA-FITC (merged); C) CFDA-FITC

High doses appeared to lead to a necrotic cell death due to mitochondrial impairment and membrane leakage. Low doses appeared to inhibit both apoptosis and cell cycle check point regulation leading to aberrant cell proliferation. Proceeding from high to low doses an hormetic zone was observed along the curve approaching the baseline which preludes to neoplastic transformation *in vitro*.

Sublethal and subcytotoxic concentrations of AFB_1 trigger apoptosis prior to induce necrosis, as assessed by the occurrence of a damage which is not recoverable, but permanent, even if the toxic insult is removed. Our results indicate almost three distinct pathways of cytotoxic response in AFB_1 treated seabream hepatocytes: necrotic cell death, apoptotic cell death, and uncontrolled cell proliferation. Such findings demonstrate that seabream hepatocytes are highly sensitive to AFB_1 exposure.

5.2 AFB_1 effects on *Vibrio fischeri*

In the last years, several biological models were used in both short- and long-term tests implementing various test organisms. The toxicological studies need for simple, inexpensive, rapid and sensitive test in order to screen an increasing number of chemicals and assess their acute and chronic effects (Fargasová, 1994; Ghosh et al., 1996; Radix et al., 2000). Bacteria are considered test organisms that offer good response to these necessity (Ghosh et al. 1996). Among the available bacterial assays, the standard Microtox® system is the most popular due to its rapidity, sensitivity, reproducibility, as well as cheap costs (Kwan & Dutka, 1990). This system measures the decrease of light emission by *Vibrio fischeri* (NRRL B-11177) after being exposed to chemicals, and it was successfully used for the toxicity evaluation of a large number of substances (Arufe et al., 2004; Fulladosa et al., 2007), contaminated water (Fernandez et al., 1995) and sediments (Narracci et al., 2009). This method is more sensitive

than other acute toxicity tests (Weideborg et al., 1997) and can be used for the prediction of chemical toxicity in other aquatic organisms (Chen & Que Hee, 1995; Zhao et al., 1995).
V. fischeri is a Gram-negative, rod-shaped, flagellate, heterotrophic bacterium recovered in marine ecosystem, with bioluminescence capability (Fig. 17). Bioluminescence is produced by bacterial luciferin-luciferase system: there is a substrate (luciferin) oxidation in presence of enzyme (luciferase). Several factors, both external and proper of bacteria, are involved in the induction or inhibition of the enzymatic transcription system in the light emission.

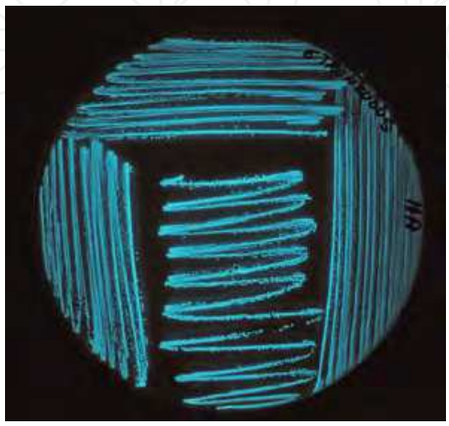

Fig. 17. *Vibrio fisheri* on Sea Water Complete (SWC) agar

The end point of Microtox® system is based on the evaluation of the light developed by this bacterium as an end product of its respiration. Any inhibition of cellular activity causes a change in the respiration rate and a corresponding variation of bioluminescence. Therefore, light emission can be considered as a signal of " health status": a toxic chemical can inhibit one of the several enzymes directly or indirectly involved in bioluminescence, leading to a gradual reduction of the light in a dose-dependent manner.
We built dose response curves measuring the bioluminescence emitted by *V. fischeri* upon AFB_1 exposure, and compared this data to those obtained from *in vitro* cell culture system, to lastly correlate the *in vitro* basal cytotoxicity data (ICs) with the validated EC_{50} value tested by the Microtox® system.
Toxicity screening was carried out by a Microtox® Model 500 Analyzer, equipped with a 30 well temperature controlled incubator, one reaction and one read well and interfaced with a PC equipped with the Microtox® Omni 1.16 software for Windows 98 for acquisition and data handling. The basic protocol employed a non-toxic control (blank) and four serial dilutions of the original sample. Reagent consisted of living luminescent bacteria grown in optimal conditions, harvested and lyophilized, and rehydrated with Reconstitution Solution to obtain a suspension of organisms for the test execution. The system measures bacterial light emission of a sample and compares it to the light emission of a control. The difference in light output is the effect of the sample on organisms.
For the toxic evaluation of AFB_1 was used the Microtox® Basic Test (BT) according to standard operating procedure (Azur, 1994). *V. fischeri* were exposed to a concentration range of AFB_1 from 0.1 pg/mL to 10 µg/mL. AFB_1 was diluted using diluent reagent for Microtox® and the Osmotic Adjusting Solution (OAS), necessary to correct the osmotic pressure of the sample to about 2% NaCl. The bacterial light emission was measured and compared to a control after 5, 15, 30 min and 3.5 h; the incubation temperature was of 15°C. An apparent

time dependent response was observed in the range 2,5-10 µg/mL, where a decrease in the light emission corresponded to a toxic evaluation from high to very high, and in the dose range 0.1-0.5 pg/mL, where a change from a non toxic assessment to a stimulatory effect was observed (Fig. 18). A clear biostimulation was also evident in the range 0.312-0.468 µg/mL, whereas a transition from stimulatory effect to non toxic evaluation was detected at 0.005 and 0.006 µg/mL. Moreover, the increase of AFB_1 concentration determined a progressive decrease in bioluminescence which was related to an enhance of toxic degree, even reaching a very high level of toxicity. This linear trend was observed starting from a dose of 1.25 µg/mL. At lower AFB_1 concentrations an alternation between non toxic assessment and biostimulation was observed. Finally, the biostimulatory effect was clearly detected at very low concentrations (0.1-0.5 pg/mL), with a time dependent increase, while the maximum biostimulation was reached for all the time exposures at 0.312 µg/mL. The mean value of EC_{50} was of 2.53 µg/mL.

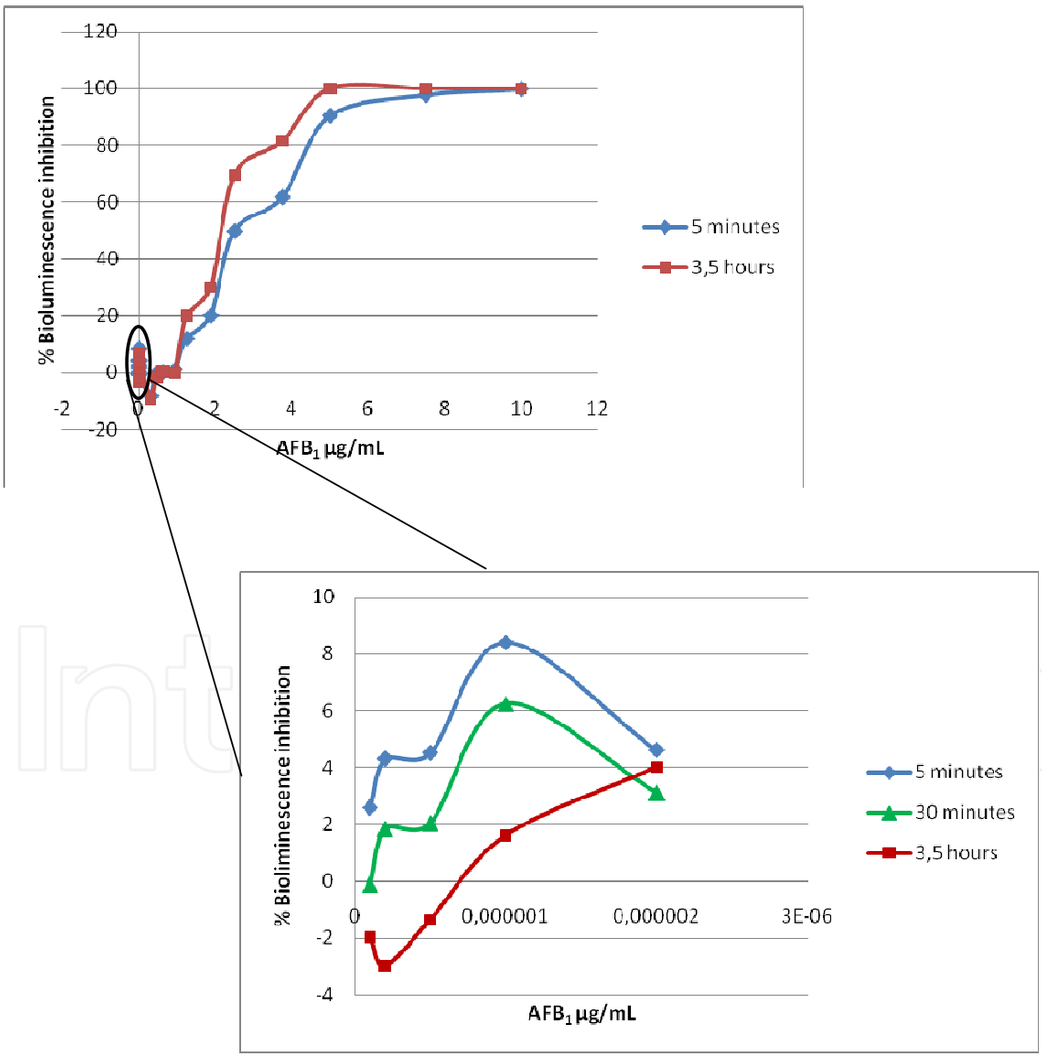

Fig. 18. Effects on bioluminescence of AFB_1 on *Vibrio fisheri* using Microtox®. Evidenced response at 0.1-2 pg/µL AFB_1 concentration.

These results shows that low concentrations of AFB_1 are able to increase luminescence intensity of bacteria compared to control, while higher concentrations are correlated with a toxic evaluation. Such evidence may be related to hormesis, which is defined as a stimulating and beneficial effect of substances at very low concentrations and harmful at high doses, or generally, a process with a biphasic trend that changes both in magnitude and in sign (Calabrese, 1999, 2002; Murado & Vázquez, 2007).

6. Conclusion

Comparing the results obtained by the two *in vitro* systems, the toxic responses are equivalent and overlapping. Therefore, this work could be considered a useful starting point for the design of new test batteries for the assessment of potentially toxic substances in aquafeed. Using an integrated approach of *in vitro* trials, during the early stages of exposure studies, can benefit *in vivo* experiments for acute and chronic exposure, determing a smaller number of better designed and targeted trials, and consequently reducing the number of sacrificed animals.

Based on the scarcity of published reports on the AFB_1 toxicity in aquacultured euryaline fish, we conclude that further research is needed in order to realize a quantitative comparison with other species. In order to provide a high level of public health protection, it would be useful to: a) investigate the bioaccumulation of AFB_1 and its metabolites in aquatic organisms of the food chain; b) investigate the quantitative correlation between AFB_1 levels found in aquafeed and the resulting residues of metabolites in fish flesh for human consumption. Overall, the finding herein presented will improve information on hazard identification and damage characterizations, providing new insights to investigate the real impact of aflatoxin B_1 on marine farmed teleosts.

Furthermore, the *in vitro* cytotoxicity cell culture based assay paralleled with the Microtox® system might provide a useful database of IC_{50}/EC_{50} values, functional to calculate or mathematically ipothesize the toxic levels of AFB_1 in other euryhaline species with unknown sensitivity. This is important for understanding the relative potencies of toxicants by using two different *in vitro* systems, and in different species.

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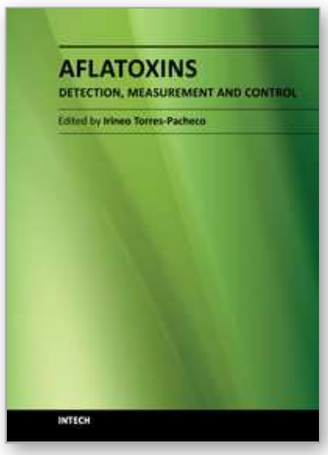

Aflatoxins - Detection, Measurement and Control

Edited by Dr Irineo Torres-Pacheco

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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical prespective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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