WORKSHOP

PARASITES FOOD QUALITY AND SAFETY: ECONOMIC AND CONSUMER HEALTH CONCERNS

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Food safety in italian fisheries and aquaculture production

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Fish consumption in Italy is around 25 Kg per capita/year; production is approximately 750,000 tons; the contribute of fisheries is 405,000 tons, while the remaining 242,000 is obtained from aquaculture and mariculture (inshore or offshore cages). Italian fishery production involves many fish species according to different techniques and geographical areas. Bivalves molluscs production represents also a relevant part of the aquaculture production This work considers food safety and risk analysis in fishery products, evidencing various production techniques, and underlining strength and weakness points.

Hygiene Package Application: outlines for an epidemiologic survey for the detection of *Anisakis spp.* in anchovy *(Engraulis encrassicholus)* catches at Civitavecchia (central Tyrrhenian sea)

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The Hygiene Package Application provides outlines for the evaluation of A*nisakis* in some fish species of commercial importance (mackerels, sprats, herrings and wild salmons). When the lack of health hazard is sufficiently proved, it is possible to reduce inspections and avoid freezing treatment of catches after the landing. Although the anchovies, *E. encrassicolus* is not listed by CE regulation 91/493/EEC, among those fish species considered of health hazard to human, however in Italy they are commonly considered as hazardous, especially for the common habit to eat them raw or almost raw.

Aim of this work is to illustrate the traditional methods of inspection to detect the presence of *Anisakis* in anchovies, and to define a procedure to be applied in a epidemiologic survey in Civitavecchia fishery products, considering: a)vfishing effort; b) total amount of landed catches; c) results about the presence of *Anisakis* spp.

Detection of some important quality reducing or potentially consumer health hazardous parasites in the flesh of fish - the Norwegian experience

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Several methods for the detection of quality reducing or potentially zoonotic parasites in the flesh of fish have been described, especially regarding anisakid nematodes such as *Anisakis* and *Pseudoterranova*. According to the EU regulation 91/493/EEC and Norwegian fish quality regulations, any visible parasites at spot tests during industrial processing of marine fish intended for human consumption, must be removed. In this respect, candling, i.e. a brief visual inspection on a light table, is the recommended method for processing plants. However, Levsen A *et al.*, 2005, J Food Prot, 68: 828-832, showed that only between 7 – 10% of the *Anisakis* larvae that are actually present in the flesh of 3 commercially important pelagic fish species, are detected by candling under simulated industrial conditions.

NIFES regularly conducts routine parasite inspections of both herring (*Clupea harengus*) and Atlantic mackerel (*Scomber scombrus*) bound for various export markets. Thus, in order to ease the routine inspection procedure for nematodes in the fish flesh, we modified the method of Karl H and Leinemann M, 1993, Arch Lebensmittelhyg, 44: 105-128, to particularly meet our requirements as to the nematode detection under field conditions onboard a fishing or research vessel. Our method is based on pressing of either whole fillets or fish sides weighing < 175g each. The pressing process is carried out on a commercially available hobby lithographic rolling device, followed by a few hours of deep freezing of the pressed fillets prior to inspection under a 366 nm UV-light source. The nematode larvae present in the frozen fillets emerge as brightly fluorescent spots within the pressed muscle tissue. Moreover, the approximate infection site of the larvae in the flesh may be pointed out as well. However, the method is unsuitable for larger fish (> 350g). In these cases, each fillet or fish side should be manually cut into thin slices (~10 mm) which then can be processed separately, or the fillets of larger fish may be pressed under a hydraulic pressing device.

The so-called 'soft flesh' condition in Atlantic mackerel is a rather newly emerging parasitic problem to the Norwegian fisheries industry. The phenomenon is characterized by a rapid *post mortem* enzymatic degradation of the fish flesh induced by the myxosporidian *Kudoa histolytica*. Recent data suggest that mainly larger mackerel (> 550g) from the northern North Sea are *Kudoa* infected; the prevalence may reach 8% in this size group (Levsen A *et al.*, submitted to J Fish Dis). Visual inspection for *Kudoa* is based on microscopic myxospore-detection of fresh or Giemsa-stained muscle smears from mackerel showing light to severe 'soft flesh' symptoms. 'Subclinical' *Kudoa* infections can only be detected by applying *K. histolytica*-specific molecular markers on various organs of a representative number of each mackerel size- or age group.

The occurrence and spatial distribution of *Anisakis* sp. in three commercially important pelagic fish stocks from the NE Atlantic, with comments on the significance to consumer safety

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The 3rd-stage larvae of the parasitic nematode *Anisakis* commonly occur in all commercially important pelagic fish species of the NE Atlantic. The parasite's life cycle involves marine mammals, planktonic crustaceans and various teleost fish species. In fish, the majority of the *Anisakis* larvae are found encapsulated on the visceral organs; however, a few worms occasionally migrate from the visceral cavity into the flesh. This phenomenon is important regarding the quality and safety of fishery products.

By applying a detection method based on screening under UV-light of flattened and frozen fillets and viscera, the abundance and spatial distribution of the *Anisakis* larvae in blue whiting (*Micromesistius poutassou*), Atlantic mackerel (*Scomber scombrus*) and North Sea herring (*Clupea harengus*) was investigated. Blue whiting were caught west to the Hebrides and Ireland in March 2006 (n=119) and 2007 (n=89), respectively, while mackerel (n=77) and herring (n=148) were caught in the northern North Sea during summer and autumn of 2006. All fish were investigated freshly onboard a commercial fishing vessel.

In both sampling years of blue whiting, the overall *Anisakis* prevalence was 100% and reached 90% in the flesh. There was a significant decrease in total abundance with increasing fish size. Moreover, there was a significant difference in larval abundance in the flesh between the smallest and the larger blue whiting, i.e. 7±5 and 4±4, respectively. The liver was the strongest infected organ carrying 69%, 50% and 43% of the total *Anisakis* burden in the smallest, medium sized and larger fish, respectively. There was also a significant decrease in larval abundance in the liver with increasing fish size. In mackerel, the overall prevalence was 97%, while 70%, 57% and 24% of the smallest (< 300 g), medium sized (300-500 g) and larger fish (> 500 g) carried *Anisakis* in the flesh, respectively. The mean abundance in the flesh was 2±3 in the smallest size group. In herring, both prevalence and abundance increased with body size. The abundance in the flesh was low, reaching a maximum of 0.5±2 in the largest size group (150-300 g). The most prominent infection site in both mackerel and herring was the pylorus area including the posterior stomach blind-sack, carrying between 57% and 81% of all larvae.

The findings suggest that the *Anisakis* infection pattern in pelagic fish is related to specific life history traits, e.g. the feeding habits of the actual species and age group, and, probably, immunological characteristics regarding the need and ability to cope with the infection. Additionally, the visceral organ topography seems to matter. For example, the relatively larger liver in small blue whiting probably "entraps" most of the larvae right after their emergence in the visceral cavity. The findings further suggest that the larvae's encapsulation site is not guided by the availability of nutrients, e.g. in the liver, but rather depends on the immunological status of each individual host.

Do anisakine nematodes facilitate bacterial spoilage of fish?

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It has previously been observed that the bacterial load of farmed fish is substantially lower compared to commercial catches of pelagic fish. Several factors may account for this, e.g. the harvesting procedure, post-catch handling and transport time.

Furthermore, several studies have shown that maricultured salmonids do not carry muscle dwelling nematodes due to the application of heat treated feed which is free of viable larvae. There is also reason to believe that the nematode abundance in the flesh of cultured non-salmonids is lower than in their wild congeners.

The intestine of fish contains high numbers of bacteria, and the population is diverse (Cahill MM, 1990, Microb Ecol, 19:21-41). Any nematode larvae that bore from the intestinal tract via the visceral cavity into the flesh of the host, may carry along bacteria on their surface or within the intestine. Bacteria from the fish intestine may also leak through the larvae's intrusion canal into the previously sterile host muscle tissue. Thus, the aim of this study is to examine the effect of muscle-penetrating nematodes on the bacterial load of pelagic fish. In the initial phase, several samples from blue whiting (*Micromesistius poutassou*) were examined by quantifying and isolating bacteria from nematode-positive and nematode-free muscle samples. The peritoneal lining in the region of sampling were washed by a cotton swab with 70 % ethanol, and samples of one gram were aseptically transferred to 9 ml Peptone water, followed by homogenizing using a sterile rod mixer. Diluted homogenate were then spread on the surface of Iron Agar Lyngby with an addition of 1 % NaCl, and incubated aerobically at 20°C for 3 days before counting the number of colony forming units (CFUs) and the proportion of H_2S producing CFUs. The latter indicates the specific spoilage flora of fish from cold waters (Gram L, 1992, Int J Food Microbiol, 16:25-39).

In the nematode-positive muscle samples (n=7), the average count of CFUs was $2.2x10^4$ (SD =$2.8x10^4$), whereas the corresponding count in nematode-free muscle samples (n=6) was $1.6x10^4$ (SD =$1.2x10^4$). The H_2S producing CFUs in muscle samples harbouring nematode larvae were on average $4.0x10^2$ (SD=$4.9x10^2$). The corresponding finding for nematode-free muscle samples was $2.2x10^2$ (SD=$3.9x10^2$).

Due to the low sample size of this initial investigation, we cannot conclude as to the possible role of muscle-invading nematode larvae as a vector of spoilage bacteria in the flesh of fish. Thus, in the next phase of the project more samples will be collected also including several other pelagic fish species. In addition to quantifying the bacteria that might be present, the DNA from the bacterial cell material obtained during cultivation will be examined by PCR/DGGE/sequencing methods. This approach may be useful to describe and compare the bacterial population in the fish intestinal content, within and in and on the nematodes and in the muscle where nematodes lodge, thus providing information on the importance of muscle-penetrating nematodes on the bacterial load and spoilage of fish.

Parasites of marine fish: which are implicated in the human health?

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Seafood is responsible of a large number of foodborne diseases occurring in the world. Many parasites including nematodes, trematodes, cestodes, and protozoa have been found in seafood. Some of these parasites may survive the food preparation process and are able to cause human infection. Anisakiasis is the human nematode infection most frequently associated with consumption of seafood. The species most commonly implicated is *Anisakis simplex*, followed by *Pseudoterranova decipiens*. Further, it is estimated that more than 50 million people are infected with food-borne trematodes worldwide. Most of these infections result from the consumption of raw or undercooked freshwater seafood. The highest prevalence is in southeast and east Asia, but increasing number of infections are being recognised in areas previously considered non-endemic.

It is now emerging that even ingestion of material from dead parasites in food is dangerous. *Anisakis simplex* causes not only direct tissue damage through invasion of the gut wall but also strong allergic reactions. Urticaria and anaphylactoid syndromes associated with the gastrointestinal infection by *A. simplex* were reported (Audicana MT *et al*, 2002, Trends Parasitol 18, 20-25). Additionally, public health risks could arise by the consumption of fish parasitized by Trypanorincha plerocercoids or *Kudoa* cysts. Particularly, *Gymnorhynchus gigas* plerorceoids occurs in the muscles of *Brama raii* and up to 100% of fishes caught for consumption may be infected. *Molicola horridus* plerocercoids are muscles dwelling species too being found often in *Xiphias gladius* with high prevalences. A few studies suggest that feeding on fish infected with *G. gigas* plerocercoids triggers the production of anaphylactic-type antibodies in both rats and mice and, by implication, possibly also in humans (Vázquez-López E *et al*, 2001, Int J Food Microbiol 64: 307–315). Further, the majority of *Kudoa* species (Protozoa: Myxosporea) infect the somatic muscle of marine and estuarine fish establishing cysts, which contain many spores. As the parasite grows, it produces proteolytic enzymes that break down the filaments of the muscle fibre and the host recognizes the presence of the parasite. Pseudocysts in fish meat, are frequently unnoticed, then infected fish could easily reach the consumer. Recently, it has been shown that BALB/c mice immunized with *Kudoa* sp. pseudocyst soluble extracts develop high levels of IgG1 and IgE antibodies, suggesting that some of components of the parasite may be allergenic. These components could potentially be responsible for type I hypersensitivity reactions after their ingestion and might thus pose a risk to human health (Martínez De Velasco G and Cuéllar C, 2003, Par Immunol 25: 449–456). At last, of particular recent concern is the presence of zoonotic protozoa in marine ecosystems in various parts of the world, especially *Cryptosporidium* and *Giardia* in shellfish (Fayer R *et al*, 2004, Vet Parasitol 126: 37-56).

Fish farming, parasite life history evolution and virulence

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During the last three decades we have seen a dramatic increase in fish farming world-wide. This trend has particularly been apparent along the Norwegian coast where the most important commercial species, salmon, increased from a few tons in 1982 to around 600 000 tons in 2006. Within the same period the wild stock of salmon has been steadily declining. The total catch of wild salmon has been around 1000 tons the last years, indicating that for each kilo of wild salmon caught about 600 kilos farmed salmon are produced. This enormous increase in local density of salmon has the potential to create a multitude of environmental problems. Of those the emergence of local disease epidemics and the dispersal of disease organisms from farms to wild stocks are among the more important concerns.

A fundamental assumption in epidemiological theory is that transmission of a parasite depends on the availability of susceptible hosts – a higher density of hosts will lead to both higher parasite abundance and to increased parasite diversity. Another consequence of increased host density, which has received less attention, is the evolutionary effect on the parasites. Rapid evolution is a common feature of parasites, due to short generation times and large population sizes, illustrated by repeated emergence and spread of drug resistance. Parasites may change their life history strategy in response to the altered environmental conditions, i.e. adapt to a situation where transmission opportunities are increased. For environmental changes towards higher availability of susceptible hosts, theory predicts that parasite genotypes which reproduce (and thus transmit) earlier, rather than over a long period of time, will be selected for. Such selection is predicted to be reflected in changes in the parasite's life history: an earlier age at first reproduction and increased early fecundity. These traits have also been associated with parasite virulence, i.e. the harm to the host caused by the parasitic infection.

Fish farming does not only change the density of susceptible hosts. Frequent slaughtering procedures and treatment against diseases may also affect parasite life histories and virulence. In this talk the possible evolutionary effects of these factors, as well as increased host density, will be discussed, using salmon lice as an example.

Antigenic activity of *Anisakis* larvae is conserved after food processing and pepsin treatments

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Problems related to ingestion of parasitized fish are rising because of new fishing grounds, growing international markets, improved transportation and storing systems. Infestation of fish by parasites may affect the commercial value of the catches and the consumer's health. Ingestion of fish parasitized with *Anisakis* larvae (L3) can cause in humans infection by the live parasite and allergy of varying intensity produced by different allergens secreted by the larvae and excreted to the host, some of which have been shown to be high-temperature, acid and pepsin resistant. Clinical symptoms due to the consumption of parasitized fish with live Anisakidae larvae have been described since decades, mainly in countries where fish is consumed raw or after treatments where the larvae remain live; however, studies relating human allergy to consumption of fish parasitized with *Anisakis* sp. have been reported only since 1990. Infection with the live larvae is prevented subjecting the fish to treatments in which the larvae are killed. However, there is scarce information on the effect of various technological or culinary treatments on the allergens from these parasites.

In order to study if antigenic activity in live and dead *A. simplex* larvae (killed by freezing according to the recommendations of the EU) is detected after different treatments, live and frozen *Anisakis* larvae were subjected to different processing treatments (heat, microwaves, vinegar, etc) and treated with acid and pepsin at different concentrations to mimic a gastric digestion. Scanning electron microscopy of the larvae showed changes in the cuticle and, in some cases, ruptures and emergence of internal tissue, the differences determined by the digestion conditions and the technological or food processing treatment given to the larvae. Nevertheless the intensity of the changes observed in live and dead larvae did not correspond with the antigenic activity of the larvae, even after pepsin digestion. Ani s 4, considered thermally stable, was detected in the larvae in all the studied treatments and conditions.

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The 2004 *Anisakis* infection in juvenile greater amberjack, *Seriola dumerili*, aquaculture seedlings imported from China to Japan and the impact on Japan's amberjack aquaculture industry

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In early spring, 2005, many juvenile amberjack aquaculture seedlings imported from southern China between November and February were found heavily infected with *Anisakis* larvae. This was the first case of mass infection with *Anisakis* in cultured fish and posed a muddle and economic loss to greater amberjack industry in Japan. In this presentation, the cause and consequence of the infection is described, as well as the occurrence itself.

The mass infection with *Anisakis* was simultaneously determined by several diagnosis laboratories for fish diseases. An emergency survey by a local government in southern Japan found *Anisakis* infection in 16 of 24 lots examined. Prevalence and intensity were 14-100 % and 30-200 worms, respectively. The worms were morphologically identified as *Anisakis* larvae Type I and later as *Anisakis pegreffii* from DNA sequences of ITS regions (Yoshinaga T *et al.*, 2006, Fish Pathol 46, 123-126). Subsequently, the identification was confirmed by allozyme analysis and mtDNA *cox-2* sequences analysis by Dr. Simonetta Mattiucci - "Sapienza" University of Rome).

Responding to the local government report, the Ministry of Health, Labor and Welfare carried out an extensive survey of *Anisakis* infection in amberjack, in which 192 of 554 amberjack examined were found to be infected and one of 134 infected fish sampled from heavily infected lots was infected in its body muscle. Based on this finding, the Ministry issued a directive to fish farmers and local governments in June 2005 that greater amberjack raised from seedlings captured in 2003 or 2004 and subsequently grown until the fall of 2004 in China, should be frozen before being brought to market, as a food safety precaution. As greater amberjack are cultured mostly for sashimi (raw fish) use and their commercial values would be largely lost after freezing, amberjack imported between autumn 2004 and winter 2005 were mostly sold at low prices as material for fish meal production. This resulted in large economic loss estimated as at least one billion JPY (= 6 million Euro).

In Japan, greater amberjack culture has mostly depended on seedlings imported from China. Generally, fingerlings below 15 cm in total length are imported in spring or early summer and raised to 3-5 kg in Japan. However, in 2004-5, juvenile fish larger than 30 cm in total length were also imported because of their low price. In China they had been fed on raw small fish (chopped or whole body), becoming infected before transportation to Japan. Since this incident, growers of greater amberjack seedlings in China have changed the fish food they use from raw to frozen fish or artificial diets, responding to the demands from Japanese fish farmers. Currently, *Anisakis*-free seedlings are being provided from China.