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Determination, prediction and extension of shelf-life

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and filleting yields even for fish that have a relatively low bacterial load, demonstrating the importance of autolytic processes.

TABLE 56
Summary of autolytic changes in chilled fish

Enzyme	Substrate	Changes encountered	Prevention/inhibition
Glycolytic enzymes	Glycogen	Production of lactic acid, pH of tissue drops, loss of waterholding capacity in muscle High-temperature rigor may result in gaping	Fish should be allowed to pass through rigor at temperatures as close to 0 °C as practically possible Pre-rigor stress must be avoided
Autolytic enzymes, involved in nucleotide breakdown	ATP, ADP, AMP, IMP	Loss of fresh fish flavour, gradual production of bitterness with Hx (later stages)	Same as above Rough handling or crushing accelerates breakdown
Cathepsins	Proteins, peptides	Softening of tissue, making processing difficult or impossible	Rough handling during storage and discharge
Chymotrypsin, trypsin, carboxy-peptidases	Proteins, peptides	Autolysis of visceral cavity in pelagics (belly-bursting)	Problem increased with freezing/thawing or long-term chill storage
Calpain	Myofibrillar proteins	Softening, moult-induced softening in crustaceans	Removal of calcium thus preventing activation?
Collagenases	Connective tissue	"Gaping" of fillets, softening	Connective tissue degradation related to time and temperature of chilled storage
TMAO demethylase	TMAO	Formaldehyde-induced toughening of frozen gadoid fish	Store fish at temperature ≤ -30 °C Physical abuse and freezing/thawing accelerate formaldehyde-induced toughening

4.1.2 Microbiological changes (Paw Dalgaard)

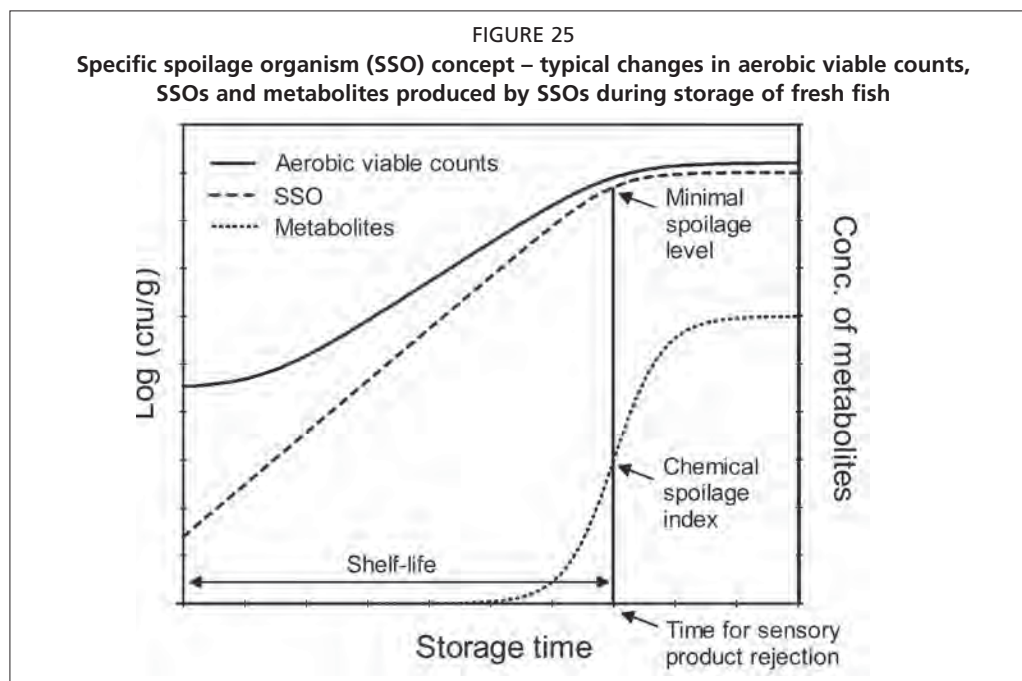
The presence, growth and activity of micro-organisms in raw material and products of finfish, crustaceans and molluscan shellfish influence spoilage and shelf-life. Assessment and management of relevant micro-organisms are important to avoid defects and problems with shelf-life. Only some of the numerous species of micro-organisms in aquatic muscle food are important for spoilage, and, during storage, a pattern of microbial growth and activity, called the specific spoilage organism (SSO) concept, is frequently observed. In newly processed, fresh or lightly preserved fish muscle, the SSOs are usually present in very low concentrations and constitute only a minor part of the total microflora. Then, during storage, the SSOs grow faster than the remaining microflora, produce metabolites responsible for off-flavours, and finally cause sensory product rejection (Figure 25). The SSOs often consist of a single or a few microbial species, whereas the microflora found in a product at the time sensory assessments indicate spoilage typically include several groups of micro-organisms. The SSOs are typically present in concentrations of about 10^7 cells/g or colony forming units per gram when sensory spoilage becomes apparent.

This section briefly describes the occurrence, growth and activity of micro-organisms in fish and seafood muscle, and illustrates how information about SSOs can be used to determine, predict and extend the shelf-life of products.

4.1.2.1 Microflora of aquatic animals from different habitats

The microflora of aquatic animals at capture or harvest depends to a large extent on the microflora of the water in which they live. Micro-organisms are found on outer surfaces (e.g. skin, gills and intestine), whereas the muscle tissue of healthy aquatic animals is sterile. The concentration of culturable micro-organisms is variable and, in general, 10^2 – 10^5 cfu/cm² are found on skin, 10^3 – 10^7 cfu/g in gills and 10 → 10^8 cfu/g in the intestines. The variable concentrations of intestinal micro-organisms are related to the aquatic animal's intake of food. Higher temperatures typically correspond to higher concentrations of culturable micro-organisms in water and on aquatic animals, whereas

water salinity has little effect on the total concentration of micro-organisms (Horsley, 1977; Liston, 1980; Cahill, 1990; Yoshimizu and Kimura, 1976; Okuzumi and Awano, 1983; Yoguchi, Okuzumi and Fujii, 1990). Regarding the effect of catching methods, trawled finfish may have 10–100 times higher concentrations of micro-organisms on skin and gills than similar fish caught by longline (Shewan, 1961).



Source: Modified from Dalgaard (2000).

The genera or groups of micro-organisms found on skin, on the outer shell and in gills of newly caught or harvested finfish, crustaceans and shellfish have been extensively studied. The dominating groups of Gram-negative bacteria are: (i) *Acinetobacter* and *Moraxella/Psychrobacter*; (ii) *Pseudomonas* and *Shewanella*; (iii) *Flavobacterium* and *Cytophaga*; (iv) *Vibrio* and *Photobacterium*; (v) *Aeromonas*; and (vi) Enterobacteriaceae. The dominating groups of Gram-positive bacteria are cocci, primarily *Micrococcus*, coryneforms and rods including *Bacillus*, *Clostridium* and lactic-acid bacteria. When data from many studies are compared, little can be concluded about the effect of water temperature and salinity or about the type of animal, for example demersal or pelagic, on the percentage distribution of genera and/or groups of micro-organisms. However, for animals in freshwater, the sodium-requiring species of *Vibrio* and *Photobacterium* are very rarely present, whereas *Aeromonas* and Enterobacteriaceae are relatively more important in those habitats. Moreover, the *Flavobacterium-Cytophaga* group seems less dominant in marine animals. Furthermore, the percentages of *Bacillus*, *Micrococcus* and Enterobacteriaceae tend to be higher in tropical than in temperate regions. The apparent lack of difference between groups of micro-organisms on aquatic animals from various habitats may result from the use of simple identification schemes relying on relatively few phenotypic characteristics (Horsley, 1977; Shewan, 1962; Liston, 1980; Cahill, 1990; Karunasagar and Karunasagar, 1991; Gram and Huss, 2000).

Owing to the high concentration of intestinal micro-organisms in aquatic animals, contamination of products during processing is important and difficult to avoid. Marine fish with a developed digestive tract have a specific gut microflora consisting of marine vibrios including *Photobacterium phosphoreum*. Fish with a simple digestive tract, e.g. immature individuals, have more complex intestinal flora that reflect the microflora in water and feed (Sera and Ishida, 1972; Yoshimizu and Kimura, 1976).

Data from several studies have shown that *Vibrio/Photobacterium*, *Pseudomonas* and Enterobacteriaceae dominate the intestinal microflora of marine fish species, whereas Enterobacteriaceae, *Aeromonas* and *Pseudomonas* dominate in the intestinal content of freshwater species. However, *Acinetobacter/Moraxella*, lactic-acid bacteria, yeasts and strictly anaerobic micro-organisms, including *Bacterioides* and *Clostridium*, can occur in high concentrations. In addition, a *Mycoplasma* phenotype has been determined in salmon by a culture-independent approach relying on extraction and amplification of 16S rDNA (Horsley, 1977; Cahill, 1990; Ringø, Strøm and Tabachek, 1995; Ringø and Gatesoupe, 1998; Spanggaard *et al.*, 2001; Holben *et al.*, 2002; Hovda *et al.*, 2007).

4.1.2.2 Microbial spoilage of aquatic muscle food

Newly caught fish and shellfish typically have a species-specific flavour that disappears after a few days of chilled storage. Further storage results in development of off-flavours, which are often ammonia-like, sulphurous, malt-like or rancid. The importance of microbial activity in seafood spoilage has been established by comparing off-flavour development in muscle pieces that were: (i) sterile, (ii) inoculated with specific micro-organisms, or (iii) naturally contaminated. These studies showed that the short shelf-life of many products is explained by their microflora and chemical characteristics.

Numerous fish and other aquatic animals of technological importance live in cold waters, and their natural microflora include psychrotolerant species able to grow readily in chilled products at temperatures above $-2\text{ }^{\circ}\text{C}$ to $0\text{ }^{\circ}\text{C}$. This explains the relatively short shelf-life of 12–18 days for many coldwater fish when stored in ice whereas the corresponding shelf-life for tropical white-fleshed fish is typically 18–35 days at $0\text{ }^{\circ}\text{C}$ (Gram, 1989; Dalgaard and Huss, 1997). In addition, the flesh of some fishes, crustaceans and molluscs contains trimethylamine oxide (TMAO), which stimulates microbial growth and activity. In general, animals from freshwater contain less TMAO than those from seawater, but considerable variation exists between species in both habitats (Hebard, Flick and Martin, 1982). *Aeromonas*, *Alteromonas*, most Enterobacteriaceae, *Shewanella* and *Vibrio* and *Photobacterium*, including all marine luminous bacteria, reduce TMAO to trimethylamine (TMA). This anaerobic respiration facilitates their growth under oxygen-limiting conditions, e.g. in vacuum-packed or modified-atmosphere packed products (Barret and Kwan, 1985; Proctor and Gunasalus, 2000). Trimethylamine contributes to the typical ammonia-like and fishy off-odours in spoiled seafoods, particularly in products with pH above ~ 6.5 (Castell and Triggs, 1955). Moreover, the post-rigor pH of finfish, crustaceans and molluscs is high compared with beef and pork. Again, this contributes to a short shelf-life. White-fleshed demersal finfish and crustaceans have a pH of ~ 6.5 to above 7, whereas pelagic, dark-fleshed fish such as tuna, mahi-mahi, mackerel and garfish, have a pH as low as ~ 5.8 . Molluscs have pHs similar to white-fleshed finfish, but they contain much more carbohydrate (2.5–5.0 percent) as compared with the < 0.5 percent for finfish and crustaceans. Consequently, a fermentative type of spoilage with decreasing pH is typical for molluscs but most unusual in other seafoods unless carbohydrates are added (Bremner and Statham, 1983; ICMSF, 1998; López-Caballero *et al.*, 2000; He *et al.*, 2002; Vasakou, Vareltzis and Bloukas, 2003). Finally, high concentrations of free amino acids are present in seafoods and metabolized by spoilage micro-organisms, e.g. arginine in shrimps, and histidine in dark-fleshed pelagic finfish (Abe, 1983; Chinivasagam *et al.*, 1998).

It is well established that many micro-organisms from seafoods produce extracellular proteolytic enzymes (Venugopal, 1990; Kobatake *et al.*, 1992). Nevertheless, seafood-spoilage micro-organisms typically produce off-flavours from substrates in muscle extractives, and proteolytic activity is not important for spoilage of fresh seafoods (Lerke, Farber and Adams, 1967; Karnop, 1982). The importance of microbial

proteolytic enzymes has primarily been evaluated for fresh fish, and further research, including on lightly preserved and semi-preserved seafoods, seems justified.

4.1.2.3 Specific spoilage organisms in groups of aquatic muscle food

Spoilage of fresh chilled and aerobically stored seafood is primarily caused by H₂S-producing *Shewanella* bacteria and *Pseudomonas* spp. This is well established (Table 57), but the taxonomy of these Gram-negative and non-fermentative rods has been changing. In fish products, H₂S-producing *Shewanella* bacteria have often been isolated as black colonies using pour plating in iron agar, and then identified as *Shewanella putrefaciens* by a limited number of phenotypic tests. It has been shown more recently that these H₂S-producing bacteria consist of a number of *Shewanella* species and that *S. baltica* is common in several types of fish products (Ziemke, 1998; Stenström and Molin, 1990; Fonnesbech Vogler *et al.*, 2005; Satomi *et al.*, 2006).

Frequently, *Pseudomonas* have not been identified at the species level, but strains similar to *Ps. fragi*, *Ps. fluorescens* and *Ps. putida* seem common in seafood, and *P. lundensis* dominates the spoilage microflora of chilled aerobically stored marine fish from Greece (Gillespie, 1981; Tryfinopoulou, Tsakalidou and Nychas, 2002; Stenström and Molin, 1990). *Pseudomonas* spp. are unable to reduce TMAO, and growth is considerably reduced under oxygen-limited conditions. Other bacteria may also influence spoilage of fresh chilled and aerobically stored seafood. Thus, *P. phosphoreum* can be responsible for TMA production and contribute to spoilage of various chilled fish stored aerobically. See Dalgaard (1998) for a review and also more recent studies of different fish species (Dalgaard *et al.*, 2006; Olafsdottir *et al.*, 2006a; Olafsdottir *et al.*, 2006b). In addition, lipolytic *Psychrobacter immobilis* can dominate the spoilage microflora in both marine and freshwater fish and, despite a low spoilage potential, they may increase the rancid spoilage of sardines (Gennari, Tomaselli and Cotrona, 1999; González *et al.*, 2000).

For fresh seafood in MAP with high concentrations of CO₂, luminous and non-luminous variants of *P. phosphoreum* are important spoilage micro-organisms (Table 57). The *P. phosphoreum* species group is heterogeneous and isolates from seafood are likely to belong to several species, including *P. iliopiscarium* (Dalgaard, Manfio and Goodfellow, 1997; Ast and Dunlap, 2005; Olofsson, Ahrné and Molin, 2007). The relative importance of *P. phosphoreum* and H₂S-producing *Shewanella* species in vacuum-packed fresh chilled seafoods probably depends on the initial concentration of the two spoilage bacteria. For shucked bivalve molluscs, i.e. mollusc meat removed from the shells, spoilage is fermentative when the product is stored under vacuum or otherwise with reduced access to oxygen, but the micro-organisms responsible, remain to be identified (Bremner and Statham, 1983; Kim, Paik and Lee, 2002; Vasakou, Vareltzis and Bloukas, 2003). Spoilage of chilled MAP seafood from freshwater or products without TMAO need further study, for example, with respect to identification of lactic-acid bacteria and the importance of *Aeromonas* spp. (Table 57). High concentrations of *Aeromonas* spp. have been found in chilled MAP seafood from tropical regions and they are likely to be the SSO (Table 57).

For chilled lightly preserved seafood, lactic-acid bacteria seem to be the most important groups of spoilage micro-organisms (Table 57). However, identification of the SSO responsible for spoilage has been complicated, and variation in product characteristics, including the initial microflora, NaCl, pH, smoke components, chemical preservatives and packaging, is probably responsible for the various spoilage patterns observed (Table 57). *Staphylococcus xylosus*, *Halobacterium salinarium* and moulds have been suggested as spoilage organisms for sun-dried tropical fish depending on storage temperature and water activity (Doe and Heruwati, 1988).

Micro-organisms in seafoods can interact in several ways, including substrate competition and metabolite inhibition (Jørgensen, Huss and Dalgaard, 2000;

Gram *et al.*, 2002). This may influence their spoilage activity and Joffraud *et al.* (2001) found mixtures of different spoilage bacteria form stronger off-odours and higher concentrations of specific metabolites than mixtures of strains from individual species. The importance of such interactions on spoilage and shelf-life of seafood warrants further study.

TABLE 57

Specific spoilage organisms in groups of fresh and lightly preserved seafood

Seafood	Typical specific spoilage organisms	Metabolites produced
Fresh chilled products stored in air		
Various species, particularly those containing TMAO ^c and with pH above 6	H ₂ S-producing <i>Shewanella</i> ^a	TMA [*] , hydrogen sulphide and other sulphur compounds, hypoxanthine
Various species, including some with little or no TMAO and low pH of about 6	<i>Pseudomonas</i> spp.	Ammonia, esters, sulphur compounds but not hydrogen sulphide
Fresh, chilled products in modified atmosphere packaging		
TMAO containing species from seawater at temperatures below ~15 °C	<i>Photobacterium phosphoreum</i>	TMA, hypoxanthine, alcohols, ketones and biogenic amines
Species from warmer waters, particularly species with little or no TMAO	Lactic-acid bacteria and <i>Brochothrix thermosphacta</i> ^b	Acetic acid, ammonia, tyramine, acetoin, diacetyl, hydrogen sulphide
Species from tropical freshwater	<i>Aeromonas</i> spp.	?
Fresh and lightly preserved products stored at ambient temperature	<i>Aeromonas</i> spp.	TMA, sulphur compounds, biogenic amines
	<i>Vibrio</i> spp. / <i>Photobacterium</i> spp. Enterobacteriaceae <i>Enterococcus faecalis</i>	NH ₃ , acetic acid, acetoin, diacetyl, tyramine
Lightly preserved and chilled products	Lactic-acid bacteria ^c and <i>Brochothrix thermosphacta</i>	Acetic acid, ammonia, tyramine, acetoin, diacetyl, sulphur compounds
Brined, spiced/gravad and smoked products, including fish roe	<i>P. phosphoreum</i> , <i>Vibrio</i> and Enterobacteriaceae ^d	TMA, biogenic amines, alcohols, aldehydes, sulphur compounds

^a *Shewanella putrefaciens*, *Shewanella baltica* and other closely related H₂S-producing Gram-negative bacteria.

^b *Brochothrix thermosphacta* is important for products in oxygen-containing modified atmospheres.

^c Include *Lactobacillus curvatus*, *Lactobacillus sake* and *Leuconostoc* spp.

^d Include *Enterobacter agglomerans*, *Hafnia alvei* and *Serratia liquefaciens*.

* TMA = trimethylamine; TMAO = trimethylamine oxide.

Source: Modified from Dalgaard (2006).

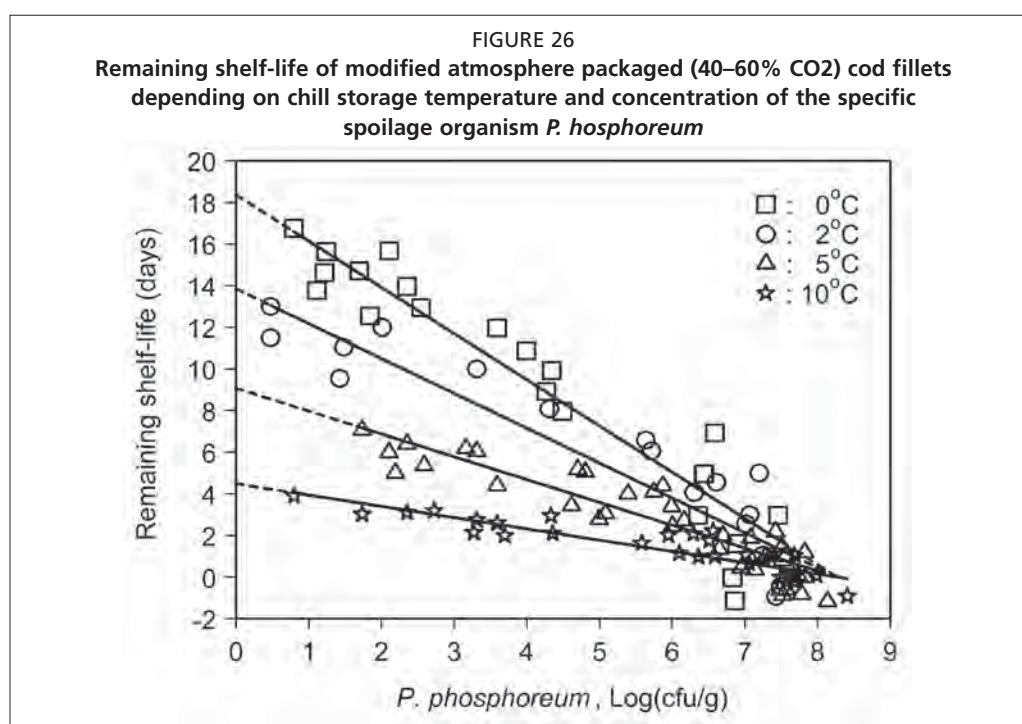
4.1.3 Determination, prediction and extension of shelf-life (Paw Dalgaard)

Indices of freshness or spoilage of seafood have been extensively studied, and it is generally accepted that there is a poor correlation between remaining shelf-life, as determined by sensory methods, and aerobic viable counts (Ólafsdóttir *et al.*, 1998). Nevertheless, the aerobic viable count is used to evaluate the hygienic status of aquatic muscle foods. Heat-labile and sodium-requiring micro-organisms are common in products from seawater and brackish waters (Dalgaard, 2006). For enumeration of these micro-organisms, pour plating with ~45 °C warm agar must be avoided as this procedure may kill a major part of the microflora. The concentration of micro-organisms in deep-water pink shrimp (*Parapenaeus longirostris*) is, for example, about 20 times higher when determined by spread plating as compared with pour plating. Moreover, micro-organisms on seafood frequently require sodium for growth and, although standard plate count agar without NaCl is recommended for many foods, this medium is inappropriate for seafood. For various fresh and lightly preserved seafoods, spread plating on pre-chilled plates of Long and Hammer's agar with 1 percent NaCl, aerobically incubated for 5–7 days at 15 °C, has been appropriate for enumeration of the dominating microflora (van Sprekens, 1974; Dalgaard, 2000;

NMKL, 2006; López-Caballero, Goncalves and Nunes, 2002). Microbiological criteria relying on mesophilic aerobic bacteria in concentrations between 10^5 and 10^6 cfu/g are included in regulatory frameworks. However, owing to the frequent dominance of heat-labile spoilage bacteria in seafood, it must not be expected that such criteria correspond to sensory spoilage.

In contrast to the situation for aerobic viable counts, close correlations (correlation coefficients between -0.929 and -0.975) have been observed between remaining shelf-life and concentrations of SSOs in different chilled fish products (Capell, Vaz-Pires and Kirby, 1998; Dalgaard, 1998; Koutsoumanis *et al.*, 1998). Thus, as shown in Figure 26 for *P. phosphoreum* in cod fillets, the concentration of an SSO can be used to estimate the remaining shelf-life at different chill storage temperatures.

For lightly preserved seafood, including cold-smoked and marinated products, simple correlations have not typically been observed between the remaining shelf-life and concentrations of any specific group of micro-organisms or any specific metabolite. However, the remaining shelf-life or sensory quality of some of these products can be related with so-called multiple-compound quality indices to concentrations of several microbial metabolites and/or specific micro-organisms (Jørgensen, Dalgaard and Huss, 2000; Leroi *et al.*, 2001).



Source: Dalgaard (1998).

Specific spoilage organisms often grow without a lag phase in fresh fish products, and this facilitates shelf-life prediction (Figure 26). In fact, kinetic models have been developed to predict growth of *B. thermosphacta*, *P. phosphoreum*, psychrotolerant *Pseudomonas* spp. and H₂S-producing *Shewanella* as a function of storage conditions (atmosphere and/or temperature). In addition, the models have been incorporated in user-friendly application software. This makes it convenient to evaluate the effect on shelf-life of product temperature profiles recorded by data-loggers. See Dalgaard (2002)⁸

⁸ The Seafood Spoilage and Safety Predictor (SSSP) software is available free of charge at: <http://sssp.dtuqua.dk>

and specific studies (Koutsoumanis and Nychas, 2000; Nuin *et al.*, 2008). Increased transportation of seafood nationally and internationally makes this type of time-temperature integration important to preventing disappointed consumers and rejections of products. Other micro-organisms, including species of *Aeromonas*, Enterobacteriaceae, lactic-acid bacteria and *Vibrio*, are also important in seafood spoilage (Table 57). For these SSOs, shelf-life models remain to be developed and/or validated in relevant seafoods. Another and more challenging future task is to predict the species of micro-organisms that become SSOs when: (i) new products are formulated; (ii) seafoods are processed by a new technology; or (iii) seafoods are stored under conditions not previously evaluated.

Extension of shelf-life is important for various aquatic-muscle foods, and both classical technologies (e.g. reduced temperature, MAP, salting, smoking and addition of antimicrobials) and newer methods (e.g. biopreservation, high-pressure processing and pulsed light) are interesting for different types of products. Inhibiting or preventing growth of an SSO results in increased shelf-life, and the extension is proportional to the growth delay. However, when growth of an SSO is markedly reduced, another SSO or another spoilage reaction (biochemical, chemical or physical) will determine shelf-life. The targeted inhibition of SSOs is interesting as product shelf-life may be extended by mild preservation methods, selected depending on properties of a particular SSO. However, the effect on shelf-life of inhibiting growth of a particular SSO must be determined using storage trials and sensory evaluation. As one example, the chilled shelf-life of frozen and thawed MAP cod, salmon and garfish is extended, compared with the fresh products, when the SSO (*P. phosphoreum*) is inactivated by frozen storage (Guldager *et al.*, 1998; Emborg *et al.*, 2002; Bøknæs *et al.*, 2002; Dalgaard *et al.*, 2006). For Nile perch at ambient temperature, spoilage is due to mesophilic motile *Aeromonas* spp. and inhibiting this SSO, using combinations of NaCl, sorbate and smoke components, resulted in a lightly preserved fish product with a marked shelf-life extension (Gram, 1991).

4.1.4 Lipid oxidation and hydrolysis (Henri Loreal)

Two important reactions can take place in fish lipids, and both lead to quality deterioration. They are oxidation and hydrolysis, and they result in the production of a range of substances, some of which have an unpleasant (rancid) taste and smell, while others contribute to texture changes by binding covalently to fish-muscle proteins. The various reactions are either non-enzymatic or catalyzed by microbial, intracellular or digestive enzymes from the fish tissues. The relative significance of these reactions depends mainly on the fish species and storage temperature.

Fatty fish are particularly susceptible to lipid degradation, which can create severe quality problems even for storage at temperatures below zero.

4.1.4.1 Lipid oxidation

The large amount of polyunsaturated fatty acid moieties found in fish lipids makes them highly susceptible to oxidation by an autocatalytic mechanism (Figure 27). The process is initiated as described below by abstraction of a hydrogen atom from the central carbon of the pentadiene structure found in most fatty acid acyl chains containing more than one double bond:



Contrary to the native molecule, the lipid radical (L) reacts very quickly with atmospheric oxygen making a peroxy-radical (LOO), which again may abstract a hydrogen from another acyl chain resulting in a lipid hydroperoxide (LOOH) and a new radical L. This propagation continues until one of the radicals is removed by

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