PART I

INTRODUCTION ON BIOTOXINS AND BIVALVE MOLLUSCS

Introduction

The area of shellfish toxins is of equal importance to consumers and producers of shellfish, as well as to regulators of food safety. In this introduction, an attempt is made to describe in brief the nature of the source organisms and the toxins involved, the difficulties encountered in protecting the consumer, and the challenge of producing safe shellfish.

First of all, it should be noted that marine biotoxins are naturally produced compounds. The term phycotoxin indicates natural metabolites produced by unicellular microalgae (protists). Most phycotoxins are produced by dinoflagellates although cyanobacteria have also been reported to produce saxitoxin (STX), and domoic acid (DA) is produced by diatoms. Some of the toxins have initially been identified in associated organisms, e.g. okadaic acid (OA), which was initially identified in the sponge *Halichondria okadaii* (Tachibana *et al*., 1981), or palytoxin (PLTX) in the soft coral *Palythoa toxica* (Moore and Scheuer, 1971). Through accumulation in the food chain, these toxins may concentrate in a variety of marine organisms, including filter-feeding bivalves, burrowing and grazing organisms (tunicates and gastropods) as well as herbivorous and predatory fish.

Human poisoning resulting from ingestion of seafood contaminated by phycotoxins has occurred in the past, and historical records as well as the habits of some populations in coastal and tropical areas show that harmful algal blooms (HABs) are naturally occurring events (Hallegraeff, 2004). In the last 30 years, HABs have attracted increasing attention from the scientific community and society. The occurrence of episodes of human poisoning resulting from ingestion of toxic seafood involving tens or hundreds of people in several areas of the world (Hallegraeff, 2004) has certainly called for more attention on HABs and their consequences on human health. The increased awareness has then supported more research efforts in the area, which are contributing to a better understanding of HABs and contamination of seafood by algal toxins. The accumulation of information in this field has led to the conclusion that a global increase in HABs and seafood contamination is under way, and more effective and complex measures to prevent human intoxications have been developed and implemented worldwide. The increased recording of occurrence of toxic algae and HABs in coastal waters in several areas in the world is certainly a result of greater attention being paid to the phenomenon. Other factors, however, are being recognized as contributing to the increasing frequency of HAB outbreaks, their appearance in areas of the world where they had not been recorded in the past, as well as the intensity and duration of HABs, with their possible consequences on seafood contamination and human intoxications/poisoning (Hallegraeff, 2004). The factors proposed as being involved in the global increase in HABs include: eutrophication of coastal waters as a consequence of increased aquaculture and fertilizer runoff from agriculture, as well as other economic activities linked to urbanization, changes in climatic conditions, and the transportation of toxic algae and their cysts from one coastal area to another as a consequence of their presence in the ballast water of ships or through the movement of shellfish stocks (Hallegraeff, 2004). Furthermore, a 2006 meta-analysis of published data and historical records provided indications that the regional loss of species diversity and ecosystem services in coastal oceans increases the occurrence of algal blooms (Worm *et al*., 2006).

Thus, HABs and the contaminations of seafood represent relevant social issues because of the problems they pose to human health, economic activities, recreation and tourism.

1. SHELLFISH TOXINS AS NATURAL PRODUCTS

Because marine biotoxins are naturally produced compounds, different algae may produce different analogues (slight variation of the main structure) and different profiles (relative composition of analogues). Therefore, similar to other anthropogenic contaminants, most groups of marine toxins have also many analogues. However, because they are naturally produced, many enzymatic systems in nature are capable of metabolizing them. This characteristic puts them in contrast to synthetic compounds such as polychlorinated biphenyls and other industrial chemicals, many of which are extremely stable compounds for which nature has no metabolic processes foreseen. Thus, between naturally produced analogues and metabolites of these, marine biotoxins constitute a vast array of bioactive chemicals.

The toxins described in the corresponding eight main chapters in Part II represent a range of marine biotoxins selected for their involvement in poisoning events or their bioactivity observed in laboratory animals in combination with their repeated occurrence in shellfish. These eight groups display a wide-ranging chemical diversity. From a natural products or biosynthesis point of view, the compounds described belong to several classes including amino acids (DA), alkaloids (STX) and polyketides (all others). Therefore, algal toxins are often referred to as small molecules. Thus, the selection of toxins excludes all compounds that are typically referred to as natural polymers (proteins, carbohydrates, nucleic acids). Indeed the molecular weight of most phycotoxins typically ranges between 300 and 1 500 daltons; nevertheless, some other compound groups, also classified as phycotoxins, such as PLTXs and maitotoxins (MTXs) are very sizeable molecules of 2 677 and 3 422 daltons, respectively. The chemical nature and molecular size classification distinguish phycotoxins from the very large group of venoms from snakes, spiders or cone snails, which are typically very potent mixtures of proteinaceous toxins. Table 1 gives an overview of some characteristics of the compound groups discussed in the specific sections.

The complex chemical nature of phycotoxins results in many difficulties in obtaining sufficient quantities of all analogues and hampers the development and validation of methods for the evaluation of their toxicity and efficient control of limits; these difficulties and their impact on consumer protection and shellfish production are further discussed below.

2. ALGAL TOXINS AND BIVALVE MOLLUSCS

Shellfish toxins are produced by algae that are consumed by bivalve molluscs as part of their natural diet. Thus, toxins are accumulated actively by shellfish and concentrated in the hepatopancreas (HP) of bivalves, their digestive organ. The factors influencing this accumulation are studied intensively. Although some toxins are accumulated very regularly by specific shellfish species in some areas, prediction of contamination levels in general remains very challenging because of a number of factors:

- Factors related to the occurrence of algae:
  - physical parameters: weather and climate-related parameters (temperature, wind, light conditions), hydrography;
  - chemical parameters: nutrient nature and availability (e.g. eutrophication), oxygen availability, anthropogenic pollution, ocean acidity;
  - biological parameters: evolution of algal community structure, occurrence of grazing and parasitic micro-organisms.

- Factors related to shellfish:
  - culture conditions: bottom- or rope-growth of mussels, subtidal or intertidal growth, water depth (and mixing of water column), maintenance of support structures (biofouling may nurture growth of toxic benthic algae);
  - filtration: feeding status, species-specific filtration rates and selectivity, micro-organisms affecting shellfish (pathogenic bacteria and viruses, algae and cyanobacteria affecting shellfish, nuisance organisms);
  - metabolism: species-specific differences, metabolic changes in bivalves as a result of seasonal variation, reproduction status and environmental stress.

TABLE 1
Physicochemical characteristics of marine biotoxins

Toxin	Chemical class	Formula	Molar weight	UV absorbance maxima (nm)	$pKa_{1,2,3,4}$	Lipophilicity
Saxitoxin	Tetrahydro-purine alkaloid	$C_{10}H_{17}N_7O_4$	299	N/A	8.1, 11.5	Hydrophilic
Domoic acid	Cyclic amino acid, 3 Carboxy groups	$C_{15}H_{21}NO_6$	311	242	2.1, 3.7, 5.0, 9.8	Hydrophilic
Okadaic acid	Polyether, Spiro-keto assembly	$C_{44}H_{68}O_{13}$	804	N/A	4.9*	Lipophilic
Azaspiracid	Polyether, Second amine, 3-spiro-ring	$C_{47}H_{71}NO_{12}$	841	N/A	5.8, N/R*	Lipophilic
Pectenotoxin-2	Polyether, Ester Macrocycle	$C_{47}H_{70}O_{14}$	858	235	N/A*	Lipophilic
Gymnodimine	Cyclic imine, Macrocycle	$C_{32}H_{45}NO_4$	507	N/A	N/R	Lipophilic
Prorocentrolide	Cyclic imine, Lac-tone Macrocycle	$C_{56}H_{85}NO_{13}$	979	N/R	N/R	Lipophilic
13dm-spirolide c	Cyclic imine, Macrocycle	$C_{41}H_{61}NO_7$	691	N/A	N/R	Lipophilic
Yessotoxin	Ladder-shaped polyether	$C_{55}H_{82}O_{21}S_2$	1 140	230	N/R, 6.9*	Amphiphilic
Brevetoxin-b	Ladder-shaped polyether	$C_{50}H_{70}O_{14}$	894	208	N/A	Lipophilic

* Fux and Hess (unpublished observations) determined chromatographically (for YTX) that pKa1 was too low to be determined chromatographically, pKa2 is given; for AZA only one pKa was determined). N/A = not applicable, N/R = not reported.

Many of the above parameters are inter-related and result in very complex and changing scenarios. For instance, duration of contamination may be related to season, and the occurrence of the same alga in summer may lead to shorter contamination episodes than its occurrence in autumn or winter.

While many of the factors affecting shellfish can be actively managed, in particular those related to culture techniques and conditions, many factors affecting the occurrence of algae are impossible to control and difficult to predict. As temperature and light conditions affect the growth of algae directly, many models for prediction are based on those parameters. However, the forecasting capability of these models remains limited because of the poor ability to forecast weather for more than one week, which is generally not sufficient warning for the shellfish industry to change harvest patterns or to relocate large quantities of shellfish. In addition, prediction models have difficulty in incorporating the biological parameters, in particular interannual variations in the phytoplankton community structure and occurrence of parasitic organisms of microalgae or conditions leading to significant cyst formation and hatching.

Significant differences in accumulation level and detoxification rate may appear between shellfish species, probably related to differences in metabolism, filtration rates and selectivity in the filtration of the algal food. These species-specific differences can

be established, and appropriate selection of species can be made to avoid the impact of certain toxic algae in specific areas.

Although for many toxins the producing algae are now known (Table 2), the causative relationship is not always clear and often requires many years of intense study. Examples of such studies are the confirmation of *Protoceratium reticulatum* as a causative organism of yessotoxin (YTX) by Satake *et al*. (1999), or the discovery of *Azadinium spinosum* as a producer of azaspiracid (AZA) (Tillmann *et al*., 2009).

TABLE 2
Toxins and their biogenetic, microalgal origins*

Toxin group	Abbreviation	Algae associated
Azaspiracid	AZA	*Azadinium spinosum*
Brevetoxin-b	BTX	*Karenia brevis*
Domoic acid	DA	*Pseudo-nitzschia spp.***
Gymnodimine	GYM	*Karenia selliformis*
Okadaic acid	OA	*Dinophysis spp.**, Prorocentrum spp.***
Palytoxin	PLTX	*Ostreopsis spp.***
Pectenotoxin-2	PTX	*Dinophysis spp.***
Prorocentrolide	PCL	*Prorocentrum spp.***
Saxitoxin	STX	*Alexandrium spp.**, G. catenatum, P. bahamense*
13-DM spirolide C	SPX	*Alexandrium ostenfeldii*
Yessotoxin	YTX	*P. reticulatum, L. polyedrum, G. spinifera*

* For a complete list of harmful algae and associated toxins, see: www.marinespecies.org/hab/index.php.
** Denotes the plural of species, i.e. several species of the indicated genus are reported to produce toxins from this group.

3. BIVALVE MOLLUSCS, A GROWINGLY USED FRESH RESOURCE

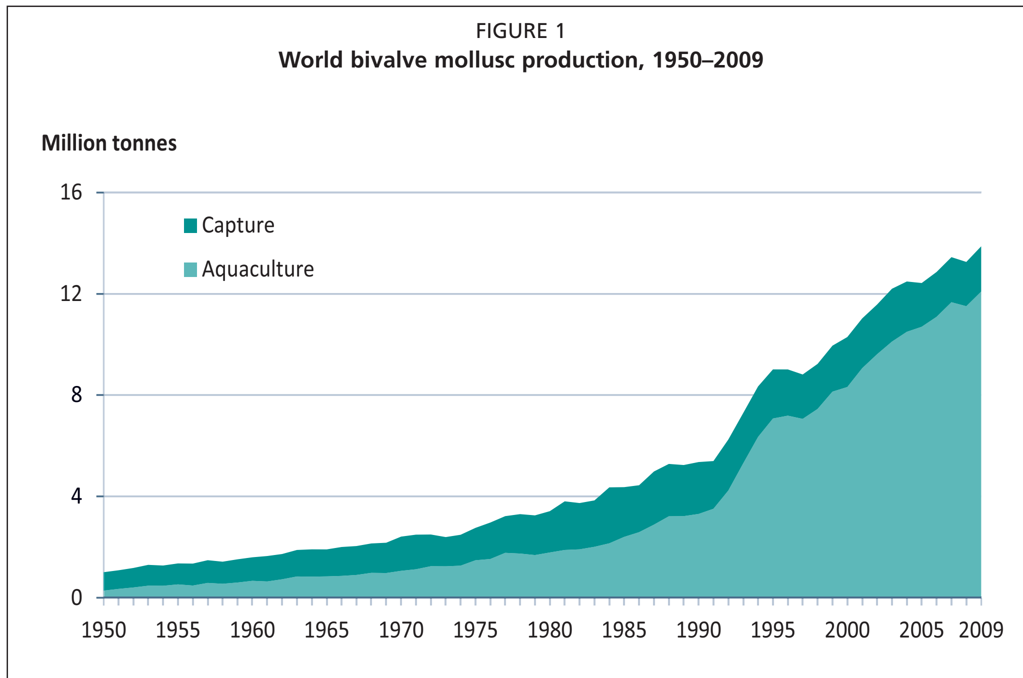
In 2007, bivalve molluscs represented almost 10 percent of the total world fishery production, but 26 percent in volume and 14 percent in value of the total world aquaculture production. World bivalve mollusc production (capture + aquaculture) has increased substantially in the last 50 years, going from nearly 1 million tonnes in 1950 to about 14 million tonnes in 2007 (Figure 1).

China is by far the leading producer of bivalve molluscs, with 9.1 million tonnes in 2007, representing 65 percent of the global molluscan shellfish production and 76 percent of the global bivalve mollusc aquaculture production. All of the Chinese bivalve production is cultured. Chinese bivalve mollusc production has skyrocketed during the last 30 years, from a mere 178 000 tonnes in 1970. The increase was particularly strong in the 1990s, with an average growth rate of 17.6 percent per year. Other major bivalve producers in 2007 were Japan (797 200 tonnes), the United States of America (764 000 tonnes), the Republic of Korea (535 000 tonnes), Thailand (386 000 tonnes), France (234 000 tonnes) and Spain (228 000 tonnes).

By species, total bivalve mollusc production in 2007 consisted of 36.0 percent of clams, cockles and arkshells, 35.2 percent of oysters, 14.3 percent of mussels and 14.6 percent of scallops and pectens, with an impressive growth in the production of oysters, clams, cockles and arkshells since the early 1990s (Figure 2).

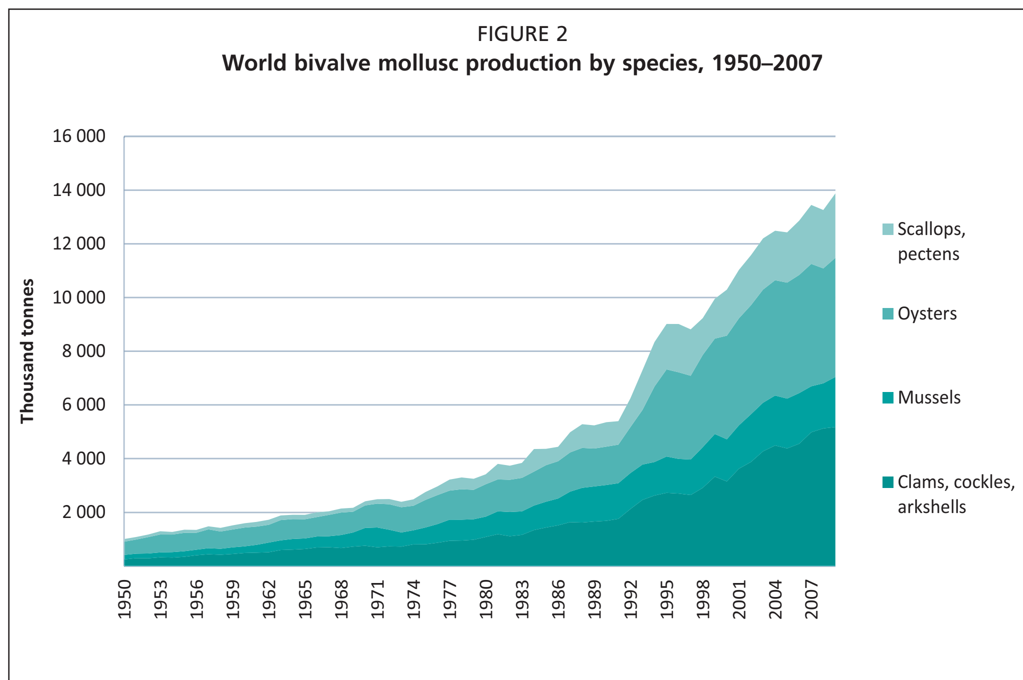
The growth in bivalve mollusc production is mainly a result of the increase in aquaculture production. World bivalve mollusc aquaculture production grew from more than 3.3 million tonnes in 1990 to nearly 12 million tonnes in 2007, with an average growth rate of 5.6 percent per year during this period. In 2007, about 85.7 percent of total bivalve production in the world (12 million tonnes) was cultured, including 97 percent of oyster production, which originated from aquaculture. This share was 95 percent for mussels, 84 percent for clams, cockles and arkshells, and 67 percent for scallops and pectens (Figure 3).

FIGURE 1
World bivalve mollusc production, 1950–2009



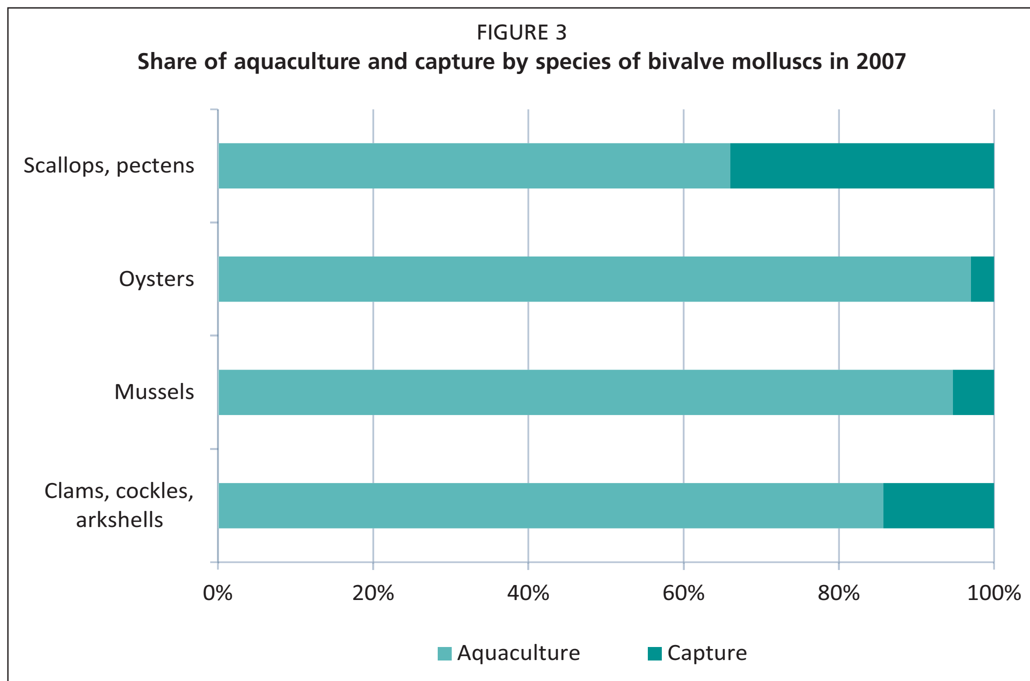
Source: FAO Fisheries and Aquaculture Information and Statistics Service, 2011a and 2011b.

FIGURE 2
World bivalve mollusc production by species, 1950–2007



Source: FAO, 2009.

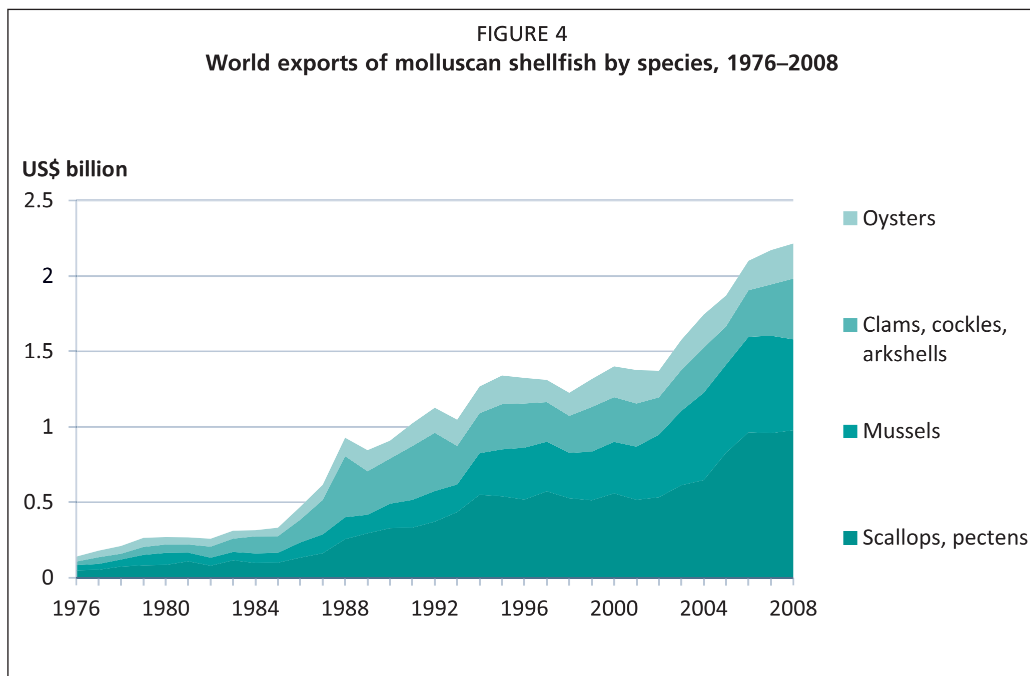
FIGURE 3
Share of aquaculture and capture by species of bivalve molluscs in 2007



Source: FAO Fisheries and Aquaculture Information and Statistics Service, 2011a and 2011b.

The increase in bivalve mollusc production was driven by international demand since the early 1990s. Total bivalve trade expanded continuously over three decades to reach US$1.87 billion in 2006. Scallops are the most important species with 38 percent of value, followed closely by mussels (33 percent). Clams and oysters are relatively less important. The share of scallops remained stable over the years, while the importance of mussel trade increased at the expense of clams (Figure 4).

FIGURE 4
World exports of molluscan shellfish by species, 1976–2008



Source: FAO Fisheries and Aquaculture Information and Statistics Service, 2010.

Bivalve molluscs, especially oysters, are consumed raw in most countries, and many are marketed as live bivalve molluscs. Because it is difficult to preserve live shellfish outside their natural environment, this also results in the need for rapid assessment of their sanitary safety.

Factors affecting the occurrence and accumulation of toxic algae cannot be controlled, and the prediction of toxic algae has severe limitations. Therefore, and because there is a very strong need to market shellfish as a live product, management practices for safe bivalve mollusc production are very specific. Official control of shellfish safety is mostly conducted on live shellfish, i.e. through regular, continuous surveillance of shellfish growing areas. This principle allows for production of live bivalve molluscs and also saves naturally contaminated shellfish from being harvested and going to waste; instead, contaminated shellfish are left to detoxify naturally in their growing waters. This practice is limited by loss of shellfish through natural mortality (typically problematic in summer) but also through specific culture techniques, such as loss of mussels from rope cultures during autumn storms. In addition, natural depuration may sometimes be prolonged over periods of several months, and thus extended closures of production zones result in significant production losses for these periods, which is generally detrimental for any business operator.

Because many algae are known to be the source organisms of toxins, phytoplankton monitoring complements official control of shellfish harvesting areas in many countries. Even though this practice is labour-intensive, it enables rapid (often voluntary) closures of shellfish production areas, thus providing additional consumer protection and reduction of production losses to the shellfish industry. Phytoplankton samples are gathered by sampling discrete water depths or the entire water column; it should be noted that the representativeness of samples is usually poor, unless the water column is well mixed; even in those cases, water masses tend to move with tides, and plankton occurrence has been described as patchy. Therefore, most managers of shellfish areas only consider information from phytoplankton monitoring to be relevant if the result indicates the presence of potentially toxic algae or blooms.

Shellfish and phytoplankton monitoring is typically conducted weekly during high risk periods, and reduced to lower frequency during low risk periods. Several countries, for instance France, Ireland and Spain, have considered higher frequency monitoring, i.e. twice weekly, for certain areas to allow for more rapid opening of shellfish areas after toxic events; however, it remains questionable whether this practice provides significant benefits compared with the additional effort spent, and whether it is in the interest of consumer safety. It should also be noted in this context that official monitoring of shellfish is often limited in terms of representativeness of the samples gathered. Often, a single shellfish sample of 5 kg shellfish is taken from areas that can cover as much as several square kilometres and contain often several hundred tonnes of shellfish. Thus, effective shellfish production area management relies heavily on time trend series, and rapid opening of areas is typically considered risky because of single samples not being considered representative. In the European Union (EU), official control therefore requires two consecutive samples to be negative before an area may be re-opened (sampled at least 48 hours apart).

Different techniques are used to increase the shelf-life of bivalve molluscs. Such techniques include freezing, cooking, canning, sterilization, cold storage, packaging under modified atmosphere and high-pressure treatments. Some of these processes mean that shellfish stay alive for longer periods, e.g. cold storage and packaging under modified atmosphere, and these processes are interesting because they increase the delay during which a product must reach the market. Hence, they also allow for more time to test the product safety before it is sold and eaten. The harsher treatments allow the shelf-life to be increased to up to two years, and thus reduce risk of toxic product reaching the market. However, these products can also be shipped much farther,

and the problem of product control has become truly global for the whole diversity of natural toxins. Therefore, in addition to the monitoring of shellfish harvesting areas, most countries also conduct official control of imported shellfish products, in particular for products from intercontinental trade.

4. SHELLFISH POISONING

The methods used for the detection of phycotoxins have historically been mainly influenced by the lack of knowledge of the exact causative agents. Without exact knowledge of all toxicologically relevant chemical entities, it is difficult to develop and validate specific, quantitative methods of analysis. In the early stages of test development, it was not even clear whether illness was caused by chemical or microbiological agents (Virchow, 1885; Wolff, 1887). Because of this lack of knowledge on the causative agents, early classifications of shellfish poisons were based on the symptoms experienced by humans following consumption of contaminated shellfish.

Four categories are distinguished:

- paralytic shellfish poisoning (caused by STX and tetrodotoxin);
- neurotoxic shellfish poisoning (caused by brevetoxins);
- diarrhoeic shellfish poisoning (caused by OA);
- amnesic shellfish poisoning (caused by DA).

Recently, AZA shellfish poisoning was discovered as a fifth category of shellfish poisoning (McMahon and Silke, 1996; Satake *et al*., 1998); the symptoms resemble those of diarrhoeic shellfish poisoning. Ciguatera and tetrodotoxin poisoning are other types of diseases associated with seafood, but these illnesses mostly arise from the consumption of fish, and are not further discussed in this context.

The exposure route for shellfish poisoning is through the consumption of shellfish. However, other routes of exposure such as through skin contact and inhalation have been observed for specific algal toxins; these include mainly brevetoxins (BTXs) and PLTXs. The main interest in this introduction is on the exposure through consumption of molluscan bivalve shellfish. From a medical point of view, it is now clear that the symptoms of these poisoning syndromes can be easily distinguished from microbiological poisoning by bacteria or viruses through the earlier onset: most bacterial or viral infections require incubation periods of 12–24 hours before sickness is experienced by shellfish consumers, while illness from shellfish toxins typically occurs as early as 30 minutes after consumption (in the case of STX or tetrodotoxin) or 2–4 hours (for most of the other compound groups).

Paralytic shellfish poisoning (PSP) has been reported worldwide (FAO, 2004). Mild symptoms include altered perception (burning or tingling sensation and numbness of the lips, which can spread to the face and neck), headache, dizziness and nausea. More severe symptoms include incoherent speech, a progression of altered perception to arms and legs, a progressive loss in the coordination of limbs and general weakness. Respiratory difficulty is a late symptom, as a consequence of muscular paralysis progressing in the whole body, and death may be the outcome of PSP by respiratory paralysis (Gessner and McLaughlin, 2008).

Brevetoxins are the causative agents of neurotoxic shellfish poisoning (NSP), which may ensue after both inhaling aerosol containing the toxins and as a consequence of ingestion of contaminated seafood. When poisoning is through the respiratory tract, the exposure usually occurs on or near the waters where a bloom of BTX producers has developed. Neurotoxic shellfish poisoning has been recorded primarily in the southeast coast of the United States of America, the Gulf of Mexico and New Zealand (FAO, 2004; Gessner and McLaughlin, 2008; Ishida *et al*., 1996). The symptoms that result from contaminated shellfish appear after minutes or hours from its ingestion, and they are more severe than those found when contaminated aerosol is involved. In the former case, symptoms are both gastrointestinal (GI) (nausea, diarrhoea and abdominal

pain) and neurological (circumoral paresthesia and hot/cold temperature reversal). In more severe cases, the muscular system (altered heart contractions, convulsions and respiratory difficulties) may be affected. Death from NSP has never been reported in humans, and symptoms resolve within a few days after exposure to the toxins (FAO, 2004; Gessner and McLaughlin, 2008).

The contamination of seafood by OA and related compounds is very common in European and Asia-Pacific countries (FAO, 2004). The symptoms of diarrhoeic shellfish poisoning (DSP) appear within one hour from ingestion of contaminated seafood, and affect the GI tract with nausea, vomiting, abdominal cramps and diarrhoea (FAO, 2004). The symptoms do not last long and usually disappear within a few days. No death has been recorded because of DSP.

The symptoms because of ingestion of DA contaminating shellfish appear within the first few hours from its ingestion, and in most severe cases, may persist for months (Perl *et al*., 1990; Quilliam and Wright, 1989; Teitelbaum *et al*., 1990). Initial symptoms affect the GI tract with nausea, vomiting, abdominal cramps and diarrhoea. These are followed by headache and other neurological symptoms, which often result in disturbances to memory, an effect that has led to the naming to this shellfish poisoning. In most severe cases, death may ensue. The neurological symptoms of amnesic shellfish poisoning (ASP) have been shown to evolve in the weeks (months) following poisoning, and anterograde memory disturbances can be accompanied by confusion, disorientation, peripheral nerve damage and changes in memory threshold.

The symptoms of azaspiracid poisoning (AZP) in humans are very similar to those described for DSP, including nausea, vomiting, abdominal cramps and diarrhoea, which disappear within a few days from the ingestion of contaminated shellfish (McMahon and Silke, 1996).

Overall, it is difficult to assess the true occurrence of shellfish poisoning in the human population, as for most diseases. Gastrointestinal disturbance as such is not a notifiable disease in many countries, and because of the rapid disappearance of the GI symptoms many shellfish consumers do not even declare the illness to a medical doctor. However, in some cases, in particular when many people fall sick from the consumption of traceable lots of shellfish, the illnesses can be properly diagnosed as shellfish poisoning. It is mostly these cases that are used in the assessment of how much toxin will start to cause symptoms in shellfish consumers. In the United States of America, in the period from 1990 to 1998, PSP outbreaks were responsible for about 20 percent of seafood-borne diseases traced to molluscan shellfish (FAO, 2004). Frequent low incidences of shellfish toxins, many of which are not reported or are under-reported, are sometimes overshadowed by large-scale incidences where several tens or hundreds of people become ill (see also Table 3).

TABLE 3
Large-scale shellfish poisoning incidents

Poisoning	No. of cases	Shellfish species	Location of illness	References
PSP	187	clams (*A. kindermanii*)	Guatemala	Rodrique *et al.*, 1990
NSP	48	eastern oyster (*C. virginica*)	North Carolina, United States of America	Morris *et al.*, 1991
DSP	164	mussels and scallops	Japan	yasumoto, Oshima and Yamaguchi, 1978
DSP	>300	blue mussels (*M. edulis*)	Norway, Sweden	Underdahl, Yndestad and Aune, 1985
DSP	>300	blue mussels (*M. edulis*)	Belgium	de Schrijver *et al.*, 2002
DSP	200	brown crab (*C. pagurus*)	Norway	Aune *et al.*, 2006
DSP	159	blue mussels (*M. edulis*)	United Kingdom	COT, 2006
ASP	107	blue mussels (*M. edulis*)	Canada	Perl *et al.*, 1990
AZP	24	blue mussels (*M. edulis*)	Ireland	McMahon and Silke, 1998

5. HISTORICAL PERSPECTIVE ON METHODS USED FOR THE DETECTION OF PHYCOTOXINS

In terms of methodology, an early breakthrough was made by Sommer and Meyer (1937) with the development of a mouse bioassay (MBA) for the detection of the agents involved in "paralytic shellfish poisoning". This procedure is based on extraction of water-soluble (hydrophilic) compounds using hydrochloric acid as extraction solvent and detection by injection of filtered crude extracts into mice. Thus, the procedure is based on the toxic response of any water-soluble, acid-stable compound in mice. As such, it is also capable of detecting DA in shellfish, albeit at levels higher than the currently regulated levels. The quantification of paralytic toxins with this method was further improved through the work of Schantz *et al*. (1957, 1958), and the method was finally validated as an official method in 1990 (AOAC, 2005). Similarly, although NSP had been reported in the United States of America since the mid-nineteenth century, formal control methods for BTXs were developed following work by McFarren (1959), and standardized as official control procedures by the American Public Health Association (APHA) in 1970 (Anonymous, 1970). Again, the APHA protocol is based on the detection of toxic principles in shellfish by injection of extracts into mice; however, in this case a lipophilic extraction solvent is used, diethyl-ether. A similar method was developed by Yasumoto, Oshima and Yamaguchi (1978) for the detection of another lipophilic toxin group: OA and analogues; the protocol in this case is based on extraction of toxins with acetone. The water-miscible nature of this extraction solvent has led to many interferences, such as low levels of STX and DA; therefore, the protocol has later been amended to include a solvent partition step between water and diethyl-ether to eliminate these water-soluble compounds from the acetone crude extract (Yasumoto *et al*., 1985). This procedure is also capable of detecting AZAs up to certain levels (Hess *et al*., 2009). Furthermore, a rat bioassay has been developed by Kat (1983). This assay detects shellfish toxins through their diarrhoeic effects in rats following oral feeding of shellfish tissues. The rat bioassay is only capable of detecting OA and AZAs, as these are the main diarrhoeic toxins. Thus, the main five toxin groups responsible for shellfish poisoning can be detected by procedures involving toxicity testing with mice or rats. However, these bioassays may not detect all toxins at the levels required for protection of public health, and generally, MBAs suffer from a number of disadvantages, including a lack of specificity, non-toxic interferences, and ethical issues around animal welfare (Combes, 2003). In addition, the bioassays for lipophilic toxins are not quantitative and thus do not lend themselves to effective monitoring practices. Moreover, the MBAs for lipophilic toxins have not been validated through interlaboratory trials and their performance characteristics are not well established.

Because of these shortcomings of bioassays, a strong need for alternative methods has emerged (Hess *et al*., 2006). In particular, there is a requirement to detect specific compound groups to be sure of the nature of a toxic event, and also to be able to detect the quantity present to be able to implement limits for these toxic compound groups.

For the development of quantitative and specific methods, the availability of pure reference compounds (the toxins themselves) is a major prerequisite. Onoue *et al*. (1931) started work on the isolation of STX analogues as the toxic principles of PSP. The efforts were significantly advanced by Schantz *et al*. (1957, 1958). However, it was not until 40 years after initial isolation efforts that the structure of STX was finally confirmed by Wong, Oesterlin and Rapoport (1971). The characterization process has been hampered for many toxins in a similar fashion because of the lack of compound mass for the studies. This lack can be understood from the fact that the organisms producing the toxin cannot always be cultured, and scientists thus rely on the natural occurrence of the compounds. In addition, the structure elucidation in early days was mostly based on chemical reaction of the compounds. The onset of more

powerful non-destructive techniques such as nuclear magnetic resonance has allowed for the characterization of smaller quantities. While several hundred milligrams were required to characterize a toxin in the 1950/1960s, nowadays 10–100 µg of compound may be sufficient to complete the structure elucidation of a novel compound. Thus, the discovery of DA as a shellfish toxin could be completed within weeks from the poisoning event (Quilliam and Wright, 1989). More typically, it takes from one to several years from the initial poisoning event to the identification of the chemical responsible for the toxic effect, e.g. for the identification of OA and AZAs (Yasumoto, Oshima and Yamaguchi, 1978; Satake *et al*., 1998). Currently, at least one reference compound is available for every compound group; however, for several toxicologically relevant analogues, there is still no certified reference compound.

With reference compounds becoming more and more available, methods alternative to the bioassays have been developed. In the early stages, many of these methods were based on liquid chromatographic (LC) separation of the toxins (gas chromatography is excluded because of the non-volatile nature of the compounds), followed by detection of ultraviolet (UV) absorbance or fluorescence of the toxins in solution (for specific methods, see compound-specific sections). However, most compounds have no specific UV absorption or fluorescence in their natural form, and thus these methods were often cumbersome because of complicated derivatization and purification steps. Since the 1990s, methods based on LC followed by mass spectrometric (MS) detection (LC-MS) have been developed, and their ease of use has led to relatively widespread application of such methods in the new millennium. The LC-MS technique was very expensive in the early stages, therefore, these methods were initially mainly restricted to developed countries (North America, Europe and Oceania). Nowadays, the technique is less expensive but still costly and sophisticated, requiring specialized staff. However, thanks to robotics, the price per analysis has decreased, thus allowing for a wider range of users to access the technique. The main advantages of this technique consist in the fact that individual analogues can be distinguished and quantified. These features result in a maximum of information on possible causative organisms and risks encountered. However, the toxic potency of each analogue must be known in order to calculate the total toxicity associated with a sample of shellfish. Other non-animal alternatives are available for some toxin groups, including methods based on antibody technology, such as dip stick tests or enzyme-linked immunosorbent assays (ELISAs). The advantage of these tests compared with LC-MS-based methods lies in their ease of use and low cost. However, these tests can only give a single response per toxin group, which lacks information on individual analogues and also on total toxicity present. For some groups of toxins, e.g. OA and STX, functional assays have been developed. Functional assays are based on the mechanism of action and thus give information on the total toxicity present, even if there is no information on individual analogues.

6. PRINCIPLES FOR THE ESTABLISHMENT OF CODEX METHODS OF ANALYSIS

The above-mentioned methods (or types of methods) can be classified for Codex purposes into four groups. Codex methods are primarily intended as international methods for the verification of provisions in Codex standards. They should be used for reference, in calibration of methods in use or introduced for routine examination and control purposes.

Codex definition of types of methods of analysis

(a) Defining Methods (Type I): A method that determines a value that can only be arrived at in terms of the method *per se* and serves by definition as the only method for establishing the accepted value of the item measured. Examples: Howard Mould Count, Reichert-Meissl value, loss on drying, salt in brine by density.

(b) Reference Methods (Type II): A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes. Example: Potentiometric method for halides.
(c) Alternative Approved Methods (Type III): A Type III method is one that meets the criteria required by the Codex Committee for Methods of Analysis and Sampling (CCMAS) for methods that may be used for control, inspection or regulatory purposes. Example: Volhard Method or Mohr Method for chlorides.
(d) Tentative Method (Type IV): A Type IV method is a method that has been used traditionally or else has been recently introduced but for which the criteria required for acceptance by the CCMAS have not yet been determined. Examples: chlorine by X-ray fluorescence, estimation of synthetic colours in foods.

The CCMAS has also laid down general criteria for the selection of methods of analysis:
(a) Official methods of analysis elaborated by international organizations occupying themselves with a food or group of foods should be preferred.
(b) Preference should be given to methods of analysis the reliability of which have been established in respect of the following criteria selected as appropriate: specificity, accuracy, precision, limit of detection, sensitivity, practicability and applicability under normal laboratory conditions, other criteria that may be selected as required.
(c) The method selected should be chosen on the basis of practicability and preference should be given to methods that have applicability for routine use.
(d) All proposed methods of analysis must have direct pertinence to the Codex Standard to which they are directed.
(e) Methods of analysis that are applicable uniformly to various groups of commodities should be given preference over methods that apply only to individual commodities.

In the case of Codex Type II and Type III methods, method criteria may be identified and values quantified for incorporation into the appropriate Codex commodity standard. Method criteria that are developed will include the criteria in section, Methods of Analysis, paragraph (c) above, together with other appropriate criteria, e.g. recovery factors.

Any Codex Commodity Committee may continue to propose an appropriate method of analysis for determining the chemical entity, or develop a set of criteria to which a method used for the determination must comply. In some cases, a Codex Commodity Committee may find it easier to recommend a specific method and request the CCMAS to “convert” that method into appropriate criteria. The criteria will then be considered by the CCMAS for endorsement and will, after the endorsement, form part of the commodity standard replacing the recommended method of analysis. If a Codex Commodity Committee wishes to develop the criteria by itself rather than allowing the CCMAS to do so, it should follow instructions given for the development of specific criteria as outlined below. These criteria must be approved for the determination in question. However, the primary responsibility for supplying methods of analysis and criteria resides with the Commodity Committee. If the Commodity Committee fails to provide a method of analysis or criteria despite numerous requests, then the CCMAS may supply an appropriate method and “convert” that method into appropriate criteria.

The minimum "approved" Codex analytical characteristics will include the following numeric criteria as well as the general criteria for methods laid down in the Analytical Terminology for Codex Use:

- precision (within and between laboratories, but generated from collaborative trial data rather than measurement uncertainty considerations);
- recovery;
- selectivity (interference effects, etc.);
- applicability (matrix, concentration range and preference given to "general" methods);
- detection/determination limits if appropriate for the determination being considered;
- linearity.

The CCMAS will generate the data corresponding to the above criteria. When a Codex Commodity Committee submits a Type II or Type III method to the CCMAS for endorsement, it should also submit information on the criteria listed below to enable the CCMAS to convert it into suitable generalized analytical characteristics:

- accuracy;
- applicability (matrix, concentration range and preference given to "general" methods);
- detection limit;
- determination limit;
- precision, repeatability intralaboratory (within laboratory), reproducibility inter-laboratory (within laboratory and between laboratories), but generated from collaborative trial data rather than measurement uncertainty considerations;
- recovery;
- selectivity;
- sensitivity;
- linearity.

These terms are defined in the Analytical Terminology for Codex Use. The CCMAS will assess the actual analytical performance of the method that has been determined in its validation. This will take account of the appropriate precision characteristics obtained in collaborative trials that may have been carried out on the method together with results from other development work carried out during the course of the method development. The set of criteria that are developed will form part of the report of the CCMAS and will be inserted in the appropriate Codex Commodity Standard. In addition, the CCMAS will identify numeric values for the criteria for which it would wish such methods to comply. The calculated repeatability and reproducibility values can be compared with existing methods and a comparison made. If these are satisfactory then the method can be used as a validated method. If there is no method with which to compare the precision parameters then theoretical repeatability and reproducibility values can be calculated from the Horwitz equation. The individual sections follow evaluation of methods according to the above-mentioned principles.

7. DIFFICULTIES IN PROTECTING THE CONSUMER

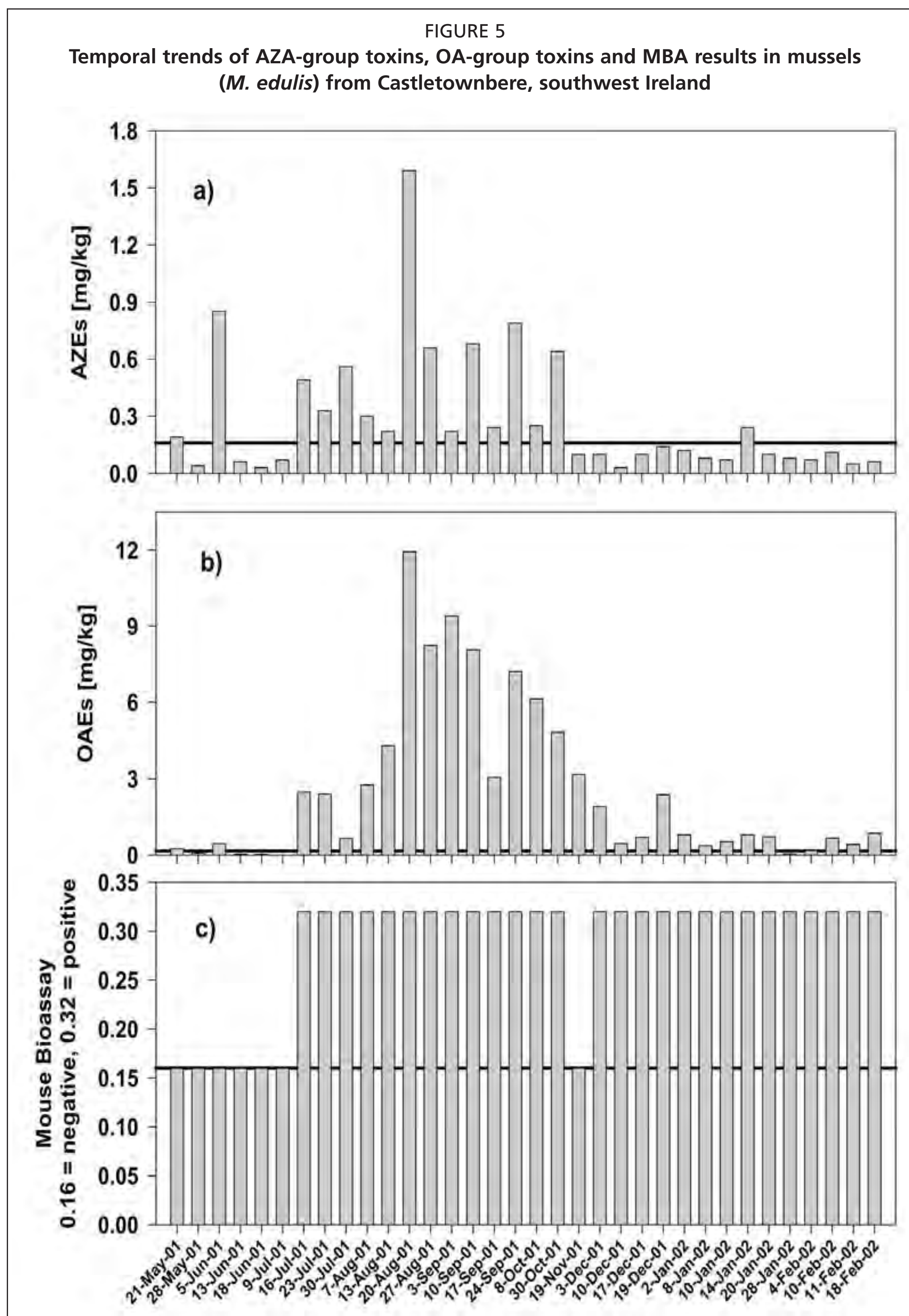
As mentioned above, shellfish toxins pose particular problems to public health protection because of a number of differences compared with other contaminants. In particular, the lack of prediction capability of the occurrence of shellfish toxins is a major factor. In addition, the requirement to produce live bivalve molluscs results in the need for continued monitoring of shellfish harvesting areas. To illustrate the difficulties encountered in protecting the consumer from the risk of shellfish toxins, several scenarios can be examined. During an algal event in the southwest of Ireland in 2001, the accumulation of toxins in blue mussels had been followed using MBA and an

LC-MS based method in parallel (Figure 5). This event could be described as a classical event, as it involves toxins that have been reported to make shellfish consumers sick at levels incurred during the event (in this case, no sickness occurred as monitoring results were known to regulatory authorities immediately, and closure of the harvest areas prevented any risk to the public). Part c of Figure 5 outlines the results of the MBA, which is the regulatory test in many countries, including Ireland. It is apparent that, using the MBA, the toxicity appears without warning, i.e. from the week of 9–16 July 2001. If the chemical monitoring, which was ongoing in parallel, had not already indicated low levels of toxins of the AZA group (see Part a of Figure 5), the area may only have been closed on 18 July 2001, i.e. 9 days from the last "non-toxic" sample date (weekly sampling plus 48 hours from the sample taken to the result obtained). This would have resulted in harvesting of the area for probably 3–5 days with high toxicity present in shellfish, which may have led to illness if these shellfish had been marketed. The rapid accumulation of shellfish toxins is a phenomenon that is often underestimated and may lead to severe public health problems as well as to significant economic losses if end-product testing is not carried out efficiently and timely.

In the same graph, it is also apparent that two shellfish toxin groups may co-occur independently; in this case OA-group toxins and AZA-group toxins. This co-occurrence may be governed by hydrographic and environmental conditions but it is not necessarily reproducible, as shown by comparison of Figure 5 with Figure 6 (2001/02 versus 2008/09). Because of the possible co-occurrence of several toxin groups, the methods used in official control of harvesting areas must be comprehensive. An MBA is capable of detecting both OA- and AZA-group toxins, as is the LC-MS based method. If an AZA-specific ELISA had solely been used in isolation, the toxicity of the OA group could have been neglected, and the shellfish growing area could have been reopened prematurely in January 2002 for the 2001 event (Figure 5). Similarly, if only a protein phosphatase assay (specific for OA-group toxins) had been used in official control of the harvesting area in Ireland in 2008, the area would have remained open in the month of June; yet, serious illness would have befallen the consumers of the shellfish because of the presence of AZA-group toxins (Figure 6). Therefore, shellfish producers and official control authorities need to know all the agents potentially causing hazards in specific areas so that methods appropriate for public health protection can be implemented.

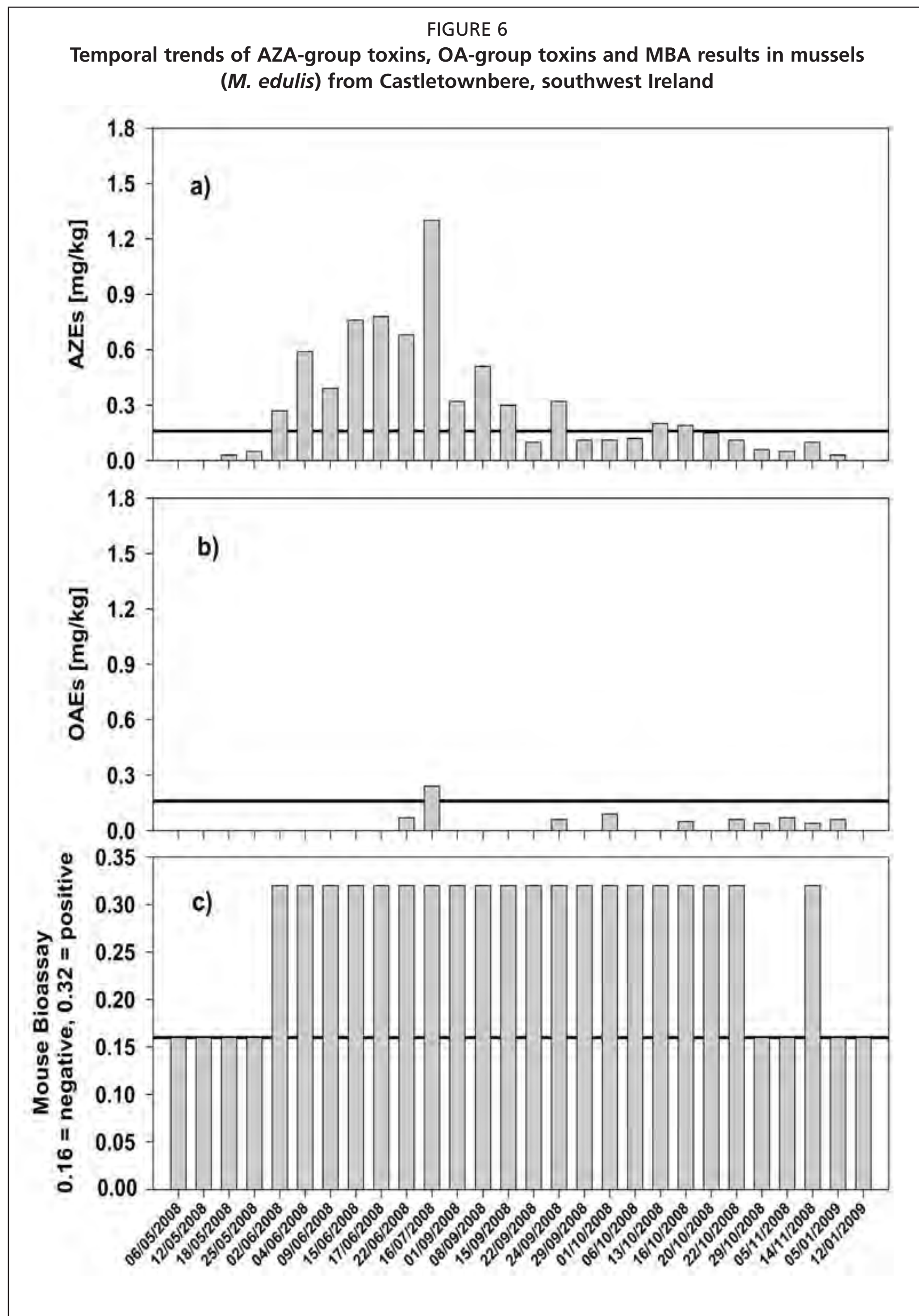
Another aspect of shellfish area management is also illustrated in Figures 5 and 6: the natural detoxification of shellfish in the growing area is significantly slower than the accumulation period. Thus, although the presence of (potentially) toxic algae may only last several days or weeks, the toxicity may persist in shellfish for many months after the algal bloom has disappeared. In this case, toxicity was still above threshold at six months after the algal appearance. These prolonged closure periods are potentially a problem for public health protection authorities, as they involve much effort in risk communication; many consumers in shellfish producing countries would be aware of the occurrence of shellfish toxicity during summer months; however, prolonged toxicity into winter months is a more recent phenomenon and requires additional efforts in managing the risks. In addition, it has been noted that detoxification rates are higher in the beginning of the detoxification, and the very slow detoxification over prolonged periods causes many problems to shellfish producers. Therefore, competent authorities also frequently face further difficulties in effectively implementing closure of production areas over these long periods.

FIGURE 5
Temporal trends of AZA-group toxins, OA-group toxins and MBA results in mussels (*M. edulis*) from Castletownbere, southwest Ireland



Notes: From May 2001 to February 2002; a) azaspiracid-1 equivalents (AZEs) and b) okadaic acid equivalents (OAEs), both determined by LC-MS and measured and expressed in whole shellfish flesh; c) MBA results for the same samples measured in HP. The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and AZA-group toxins. Arbitrarily, and for visualization purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg.
Source: Figure adapted from Hess *et al.*, 2003.

FIGURE 6
Temporal trends of AZA-group toxins, OA-group toxins and MBA results in mussels (*M. edulis*) from Castletownbere, southwest Ireland



Notes: From May 2008 to January 2009; a) azaspiracid-1 equivalents (AZEs) and b) okadaic acid equivalents (OAEs), both determined by LC-MS and measured and expressed in whole shellfish flesh, c) MBA results for the same samples measured in HP. The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and AZA-group toxins. Arbitrarily, and for visualization purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg.
Source: Figure created from data available online from the Marine Institute, at www.marine.ie, accessed on 29 July 2009.

8. CHALLENGES IN THE PRODUCTION OF SAFE SHELLFISH

The challenges in the production of shellfish are multiple. Production efficiency is related to environmental parameters and local conditions as well as production mode and implementation. In addition, natural factors such as summer mortality, shellfish pathogens and storm conditions may significantly reduce annual production by up to 80 percent in some years. Further food safety risks also arise at a high level from microbiological human pathogens, such as viruses (mostly norovirus and hepatitis A virus) and bacteria (in particular vibrios). While official microbiological classification of harvest areas results in a continuous cost of producing safe live bivalve molluscs (in Class B or moderately polluted areas), peak occurrences of pathogens may also lead to unpredictable closures reducing the productivity of a given harvest area.

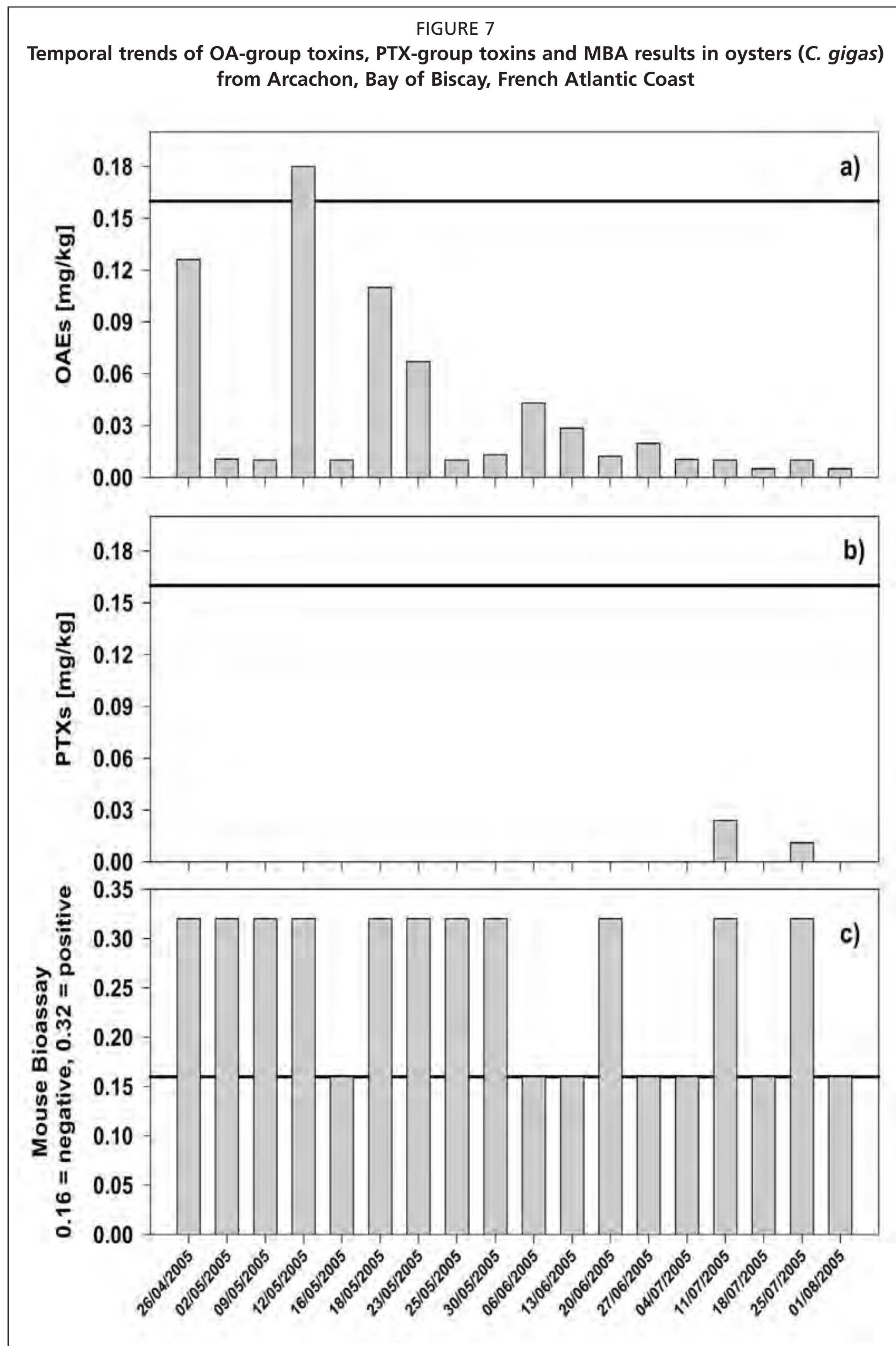
With the exception of oysters, bivalve molluscs as a raw product are considered low-cost food in most countries; typical prices being less than €1 000/tonne (about US$1 400/tonne) at production level. This is very much in contrast with many other foods, e.g. crustaceans such as lobster or crab, which may easily yield five to ten times higher income to producers, even though meat yield may be very similar. In view of this low value, end-product testing of shellfish safety becomes a major challenge. In the production of shellfish, the price for a single end-product test may be around €100–200 (about US$140–280). As algae often occur in thin layers, and with patchy structures, production lots may often be contaminated very inhomogeneously. Depending on what would be considered a representative number of tests to conduct per production batch, proper end-product testing could cost as much as 10 percent of the total product value.

An in-depth review of the Irish rope mussel sector (BIM, 2006) can serve as an example of the economic status of the bivalve mollusc sector. This report indicates that profit margins varied between 1 and 8 percent on average (for the years 2003—2005), depending on the production year. The most important one factor influencing productivity and profitability of the rope mussels sector are marine biotoxins.

This also means that in years of high biotoxin occurrence, some producers will invariably make a loss. In addition, the structure of the shellfish industry is still dominated by a large percentage of small and medium-sized enterprises. Therefore, if biotoxins occur at high levels in consecutive years, some small producers will risk bankruptcy because of lack of income.

Apart from these economic boundaries in which the shellfish producers operate, there are also challenges associated with emerging toxins and the type of testing used in official control. As mentioned above, official control has historically relied upon animal testing to assess the toxicity present in shellfish samples. Over the years, the compound groups responsible for causing shellfish poisoning have been identified, yet because of the lack of pure compounds, toxin-group specific methods have not been implemented as official methods for most toxin groups (apart from DA and STX). The scenario shown in Figure 7 exemplifies differences in the interpretation of toxin events, depending on the method used in the official control of harvesting areas. In this area (Arcachon, Bay of Biscay, French Atlantic Coast), OA-group toxins known to cause human poisoning exceeded the regulatory limit only during one week during the spring of 2005. Other toxin groups have also been monitored, including pectenotoxins (PTXs), which occurred at very low levels, and always below regulatory levels. In addition, all other regulated lipophilic toxins (YTXs and AZAs) and known non-regulated bioactive compounds (gymnodimines and spirolides) were either totally absent or present at levels more than tenfold lower than the regulatory limits. Yet, the MBA as the reference test gave many positive results for the area over the whole summer period. In fact, the area could not be opened in summer 2005 because of the sporadic occurrence of positive results.

FIGURE 7
Temporal trends of OA-group toxins, PTX-group toxins and MBA results in oysters (*C. gigas*) from Arcachon, Bay of Biscay, French Atlantic Coast



Notes: From April 2005 to August 2005; a) okadaic acid equivalents (OAEs) and b) sum of PTXs (PTX2 and PTX2sa), both determined by LC-MS (measured in HP and expressed in whole shellfish flesh); and c) MBA results for the same samples (measured in HP). The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and PTX-group toxins. Arbitrarily, and for visualization purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives are represented as 0.32 mg/kg.
Source: Figure created from data from IFREMER (French national monitoring programme REPHY); extracted 27 July 2009.

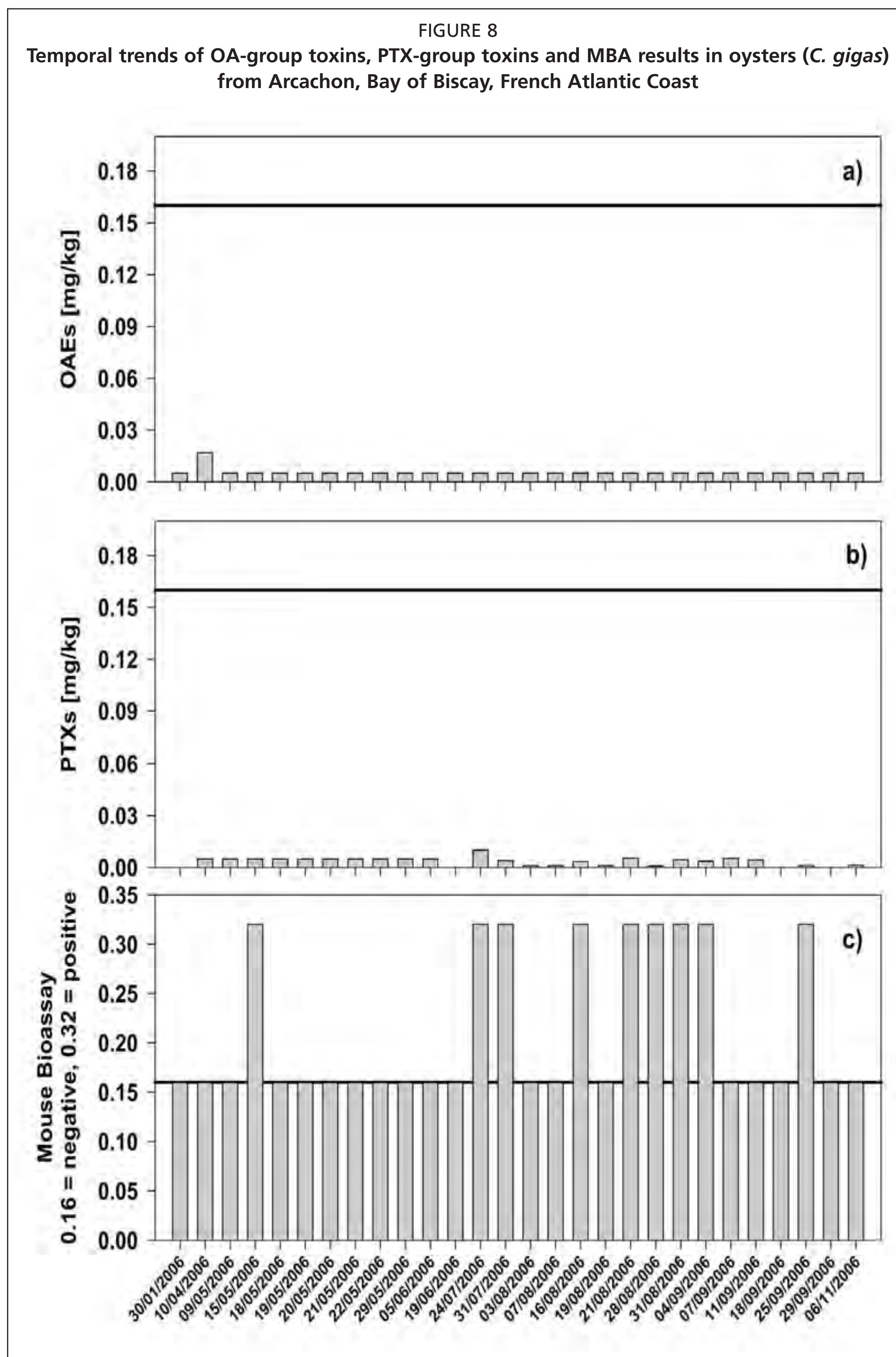


FIGURE 8

Temporal trends of OA-group toxins, PTX-group toxins and MBA results in oysters (*C. gigas*) from Arcachon, Bay of Biscay, French Atlantic Coast

Notes: From January 2006 to November 2006; a) okadaic acid equivalents (OAEs) and b) sum of PTXs (PTX2 and PTX2sa), both determined by LC-MS (measured in HP and expressed in whole shellfish flesh); c) MBA results for the same samples (measured in HP). The thick black line in each of the three graphs represents the regulatory limit in the EU at the time, i.e. 0.16 mg/kg for both OA- and PTX-group toxins. Arbitrarily, and for visualization purposes only, MBA negative results are represented as 0.16 mg/kg values whereas MBA positives *are* represented as 0.32 mg/kg.

Source: Figure created from data from IFREMER (French national monitoring programme REPHY); extracted 27 July 2009.

These positive results of the MBA may be related to yet unknown toxins of public health relevance, or they may be because of interference from bioactive compounds that are not relevant to public health. Thus, in a regime that had been based on chemical analysis (by LC-MS), production would have continued after a three-week closure period (one-week toxin levels exceeded regulatory limits, and two consecutive clear tests are required to re-open an area). The following year showed an even more dramatic picture where MBA results were again sporadically positive between May and September, while all lipophilic toxins known to occur in this area (OA, AZA, PTX, GYM, SPX, YTX) were well below the threshold expected to result in positive results in MBAs (Figure 8). The situation in Arcachon in 2006 had been further complicated by the fact that anecdotal evidence provided by oyster producers from the area suggested that consumption of these oysters did not result in acute human illness. Therefore, the question remains on how to manage areas where parallel tests yield contradictory results. The interaction between risk evaluation and risk management as integral parts of risk analysis are outlined in the next section.

9. RISK ANALYSIS PRINCIPLES AND ITERATION OF RISK ANALYSIS PROCESS FOR PHYCOTOXINS

Codex has laid down working principles for the risk analysis of food stuffs in a guideline (Codex Alimentarius, 2007) to clarify the approach proposed to governments. In these guidelines, a clear role is attributed to each of the following three integral components of the process:

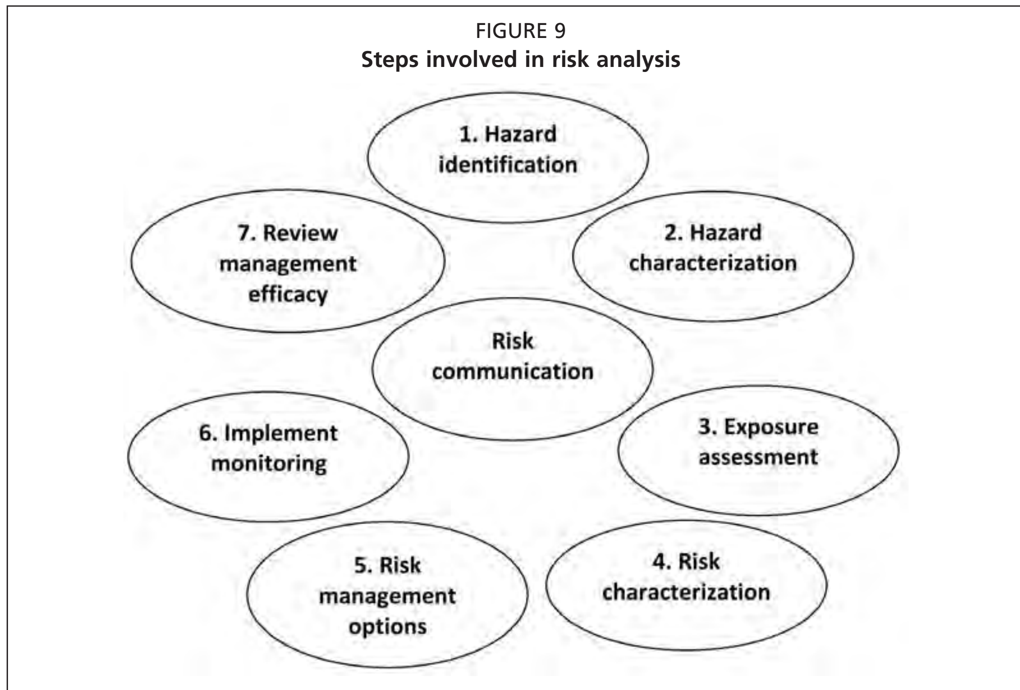
1. risk assessment or evaluation;
2. risk management;
3. risk communication.

This three-pronged approach to risk analysis (Figure 9) should be applied consistently in an open, transparent and documented manner. In addition, risk analysis should be evaluated and reviewed in light of newly generated scientific data. There should be a functional separation of risk assessment and risk management to the degree practicable in order to ensure the scientific integrity of the risk assessment, to avoid confusion over the functions to be performed by risk assessors and risk managers, and to reduce any conflict of interest. Risk communication is required for the sake of consumers and food producers but also to improve understanding between risk assessors and risk managers, for instance to clarify elements of uncertainty in a risk assessment to risk managers. Risk assessment should be structured as a process including the elements of hazard identification and characterization, exposure assessment and risk characterization. Risk management also follows a structured approach including specific steps, such as preliminary activities, evaluation of risk management options, implementation, monitoring and review of the decisions taken.

While these general principles make the Codex approach very clear, it must be noted that specific risk analyses are far from trivial, in particular because of the frequent lack of data on toxin analogues, relative toxicities, exposure and epidemiology. This lack of data often makes risk assessments provisional and requires frequent review of the assessment and the management options derived. This fact has also been recognized by Codex and, therefore, the iterative character of the risk analysis process has been stressed in the guidelines.

In the following sections, a few important steps are addressed to exemplify why a risk analysis process may often take a number of years before a satisfactory process for managing the risk is achieved.

FIGURE 9
Steps involved in risk analysis



Notes: Risk assessment includes steps 1–4; risk management includes steps 5–7. Risk communication ensures understanding of all steps by all stakeholders. The process is considered iterative as new information becomes available.

9.1 Hazard identification and characterization

When a new shellfish toxin emerges in an area of shellfish production, it is not easy to identify this hazard. Initially, there will be customer complaints about food in general; it needs to be established that shellfish were the likely source of the customer complaint. Once it has been established that bivalve molluscs were actually at the origin of the illnesses reported, medical doctors need to establish whether the nature of the illness is of microbiological or chemical origin. Because microbiological contamination of shellfish often generates similar symptoms of diarrhoea, vomiting and sickness, it is not necessarily clear that a novel toxic agent is involved. As mentioned above, the most important information comes from the epidemiological reports on the onset of the sickness, because microbiological contaminations typically require incubation periods of 24 hours before symptoms develop (some, such as hepatitis virus, may cause illness as late as 4 weeks after consumption of the shellfish). Subsequently, symptoms will need to be examined for their specificities, including neurological poisoning symptoms, memory loss, inversion of hot and cold sensation, etc.; all these symptoms could arise from known shellfish toxins. However, often, the unspecific symptoms of diarrhoea, vomiting and sickness outweigh specific symptoms in most customers, in particular if the toxin has not occurred at very high levels.

As soon as complaints are traced back to shellfish, both shellfish producers and government officials will trace back the products to their production site and will gather additional data available from routine monitoring of these sites for microbiological agents and (potentially) toxic phytoplankton. Only if it can be established that no known agents from this area could have caused the disease, work can begin on the identification of a new hazard. Most often, at the first occurrence of a new agent, the time between illnesses reported and the time when it was established that a new toxin is probably the cause of the illness is so long that there is some likelihood that the agent may again have naturally depurated from the shellfish and the shellfish growing in the area at that time may not be contaminated anymore. However, sometimes this is not the case, and contaminated shellfish may be obtained from large production lots if

these are not yet all consumed or from the area in which the shellfish are grown. For instance, when a new toxin was suspected from mussels in Killary, Ireland, 1995, the contamination had happened in autumn (September/October), and when government officials collected shellfish from the area in November, contamination was still sufficiently high (McMahon and Silke, 1996) to identify the toxin involved, probably because of slow detoxification in late autumn (colder water temperatures, fewer non-toxic algae present as food for mussels). However, because of a lack of specialized scientists in Ireland, it took the effort of international collaboration with experienced teams in Japan to identify the toxin within two years of the initial discovery of the illness (Satake *et al*., 1998). In times when virtually all scientific activities are accounted for through publicly funded projects, it is not always easy to mobilize scientific capacity, even if it is available in the country where the illness occurs. These logistic issues around finances and scientific capacity often result in an emerging hazard not being identified for years.

As mentioned above, natural toxins typically occur as mixtures of analogues. Often, the toxin-producing phytoplankton species produce two or more main analogues of a toxin, and these are further metabolized by shellfish into a multitude of analogues. As it is not guaranteed that the most abundant metabolite is also the most toxic one, hazard identification is also about the identification of all relevant analogues and about the assessment of their relative toxicity. For instance, in the case of AZAs, it should be noted that two of the three currently regulated analogues were only discovered and crudely characterized for their toxicity a further two years later (Ofuji *et al*., 1999). By 2008, 20 analogues of this toxin group were known (Rehmann, Hess and Quilliam, 2008) and metabolism in shellfish started to be understood (McCarron *et al*., 2009).

The identification of the toxin alone is only the first step of a full hazard identification. In the case of shellfish toxins, it is important to identify the biological source organism of the toxin, mostly unicellular algae (diatoms and dinoflagellates). Once these producing organisms are known, the surveillance system can observe the frequency of their occurrence and the conditions leading to toxin production, such that the full extent of the occurrence of the toxin can be understood. In the case of AZAs, for instance, it took 12 years from the discovery of the toxin until one AZA-producing organism was discovered (Tillmann *et al*., 2009). Again, this work was far from trivial because it involved the discovery of a new species and a new genus in an area completely different (North Sea) from the area of the initial discovery of the toxin (Irish Atlantic coast).

In order to fully characterize the hazard deriving from a new toxin (group), it is also necessary to understand the toxicity of the chemical substances involved. Many questions need to be answered for the characterization of the hazard:

- Is it the substance itself (produced by the alga) that is toxic or is it the metabolites in shellfish or in humans that cause the actual toxic effect?
- What is the toxic effect? Is it only digestive trouble, or are there other more grave or more subtle effects?
- Is there only acute toxicity or are there also long-lasting chronic effects?
- What is the molecular mechanism of action of the toxin?

Depending on the availability of the compounds involved and the complexity of the toxicity, it may take several years, sometimes decades to answer the above questions. In fact, while for some toxin groups one mechanism of action is known (BTX, DA, OA, STX), it remains questionable whether other mechanisms of action do not also contribute to the overall toxicity and need to be considered in the risk assessment. For other toxin groups, such as YTX, cyclic imines and AZA, the mechanism of action is not yet clarified, although these toxins were discovered in the 1980s and 1990s.

The problems of identification of the compounds and the characterization of their toxic effects are closely intertwined through the need for isolation of large quantities

of highly purified compounds. In the identification of the molecule, high purity is required to identify unequivocally the structure of the molecule, mostly through mass spectrometric and nuclear magnetic resonance techniques. In toxicological evaluation, the purity of the compound is important to associate a certain level of toxicity with one analogue only to establish its relative toxicity; if other analogues remain as impurity, the assessment of this particular analogue may be strongly falsified, because even structurally closely related analogues may differ 100- or 1 000-fold in their toxic activity. The efforts required to identify a completely novel bioactive compound are often heavily underestimated. As a rule of thumb, it should be noted that preparative isolation procedures typically only recover about 5–10 percent of the compound originally present in shellfish. Even though shellfish toxins typically cause illness at the milligram per kilogram level, tenfold larger concentrations are often required to purify novel compounds efficiently. Moreover, because the mode of action is usually not known at the beginning, the isolation procedure often relies on insensitive animal testing for the activity-guided fractionation and isolation of the toxin, thus consuming the toxin during its isolation. In many cases, the collection and dissection of hundreds of kilograms of shellfish are required in order to recover sufficient toxic material for successful identification of the compound. Toxicological evaluation requires significantly higher quantities of the compound because many experiments need to be repeated in animals, and thus for most shellfish toxins, information on chronic effects is still not available.

9.2 Exposure assessment

The assessment of the extent to which a single consumer or a population is exposed to a specific shellfish toxin is derived from two main variables: consumption of shellfish and occurrence of the shellfish toxin.

Consumption of shellfish, as of any other commodity, is mostly determined through consumption surveys. A difficulty with bivalve molluscs is the multitude of portion sizes consumed that changes from region to region, consumer to consumer and by shellfish species. A difficulty in interpreting many surveys is the fact that surveys were poorly designed in the definition of the portions consumed: it is not always clear whether the weight reported refers to the shellfish with or without their shell and intervalvular fluid, or whether it refers to the shellfish flesh consumed. In addition, it is not clear whether the weight refers to the raw weight or to the cooked weight. These difficulties result in significant uncertainty in the exposure assessment and in the evaluation of the dose that made people sick (see next section).

The occurrence of the new shellfish contaminant is not well known at the stage of its appearance. This lack of knowledge is one of the major reasons for the iterative character of risk analysis. As described above, under hazard identification and characterization, it may take several years before the chemical structure and behaviour of a new toxin are actually known. Toxin-specific methods can only be developed at that stage, and the concentration levels of the toxin may only be known after some time. If shellfish tissues from the poisoning event are retained and well preserved through freezing at low temperatures, it may later be possible to establish retrospectively the concentration of the toxin in the shellfish that had made people ill. However, more often than not, this is not the case. Therefore, it is most important that one of the risk management options for the establishment of the extent of the problem includes regular monitoring of the toxin responsible for causing the sickness encountered. The methods used for such monitoring are typically not yet fully validated through interlaboratory trials because there is no large-scale interest in working on a toxin as long as the extent of the problem has not been established.

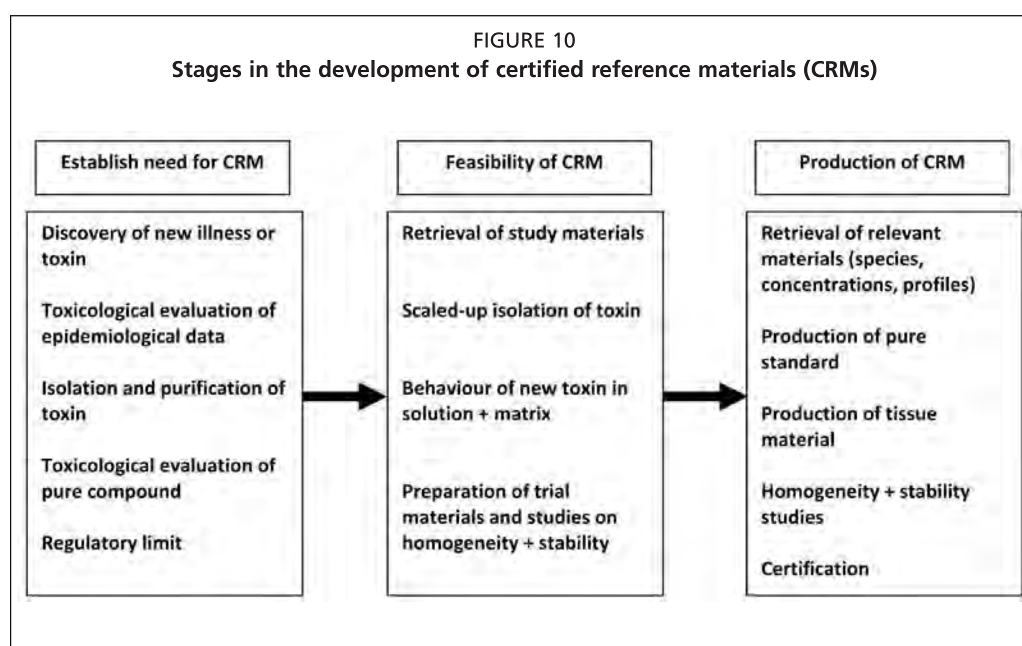
Once monitoring reveals that the compound does occur with some regularity, and once toxicological evaluation has clearly established that the compound poses a

significant risk, work on methods needs to be intensified. Such studies include the establishment of quality control tools referred to as reference materials. Steps involved in the making of a reference material are outlined in Figure 10.

Once reference materials are available for a toxin group (including certified standards and shellfish tissue reference materials), a method typically progresses to the stage that method validation may proceed. Ideally, a method is characterized through an interlaboratory study, an exercise for which further reference materials are required.

Best data on occurrence of shellfish toxins are obtained after methods have been validated and regular monitoring is ongoing with quantitative, toxin-specific methods, such as liquid chromatography with ultraviolet absorption detection (LC-UV) for DA or LC-MS for AZAs. However, for many lipophilic toxins, the reference method has remained the MBA for lipophilic toxins since the introduction of EU legislation on marine biotoxins in 1991. The existence of this reference method means that most regular monitoring is carried out with this method and that neither quantitative nor toxin-specific data are available from this surveillance. Therefore, the situation for many lipophilic toxin groups is very complex to assess, and exposure assessment relies on parallel testing that is sometimes carried out in countries that have recognized the value of quantitative toxin-specific methods beyond regulatory reference tests.

FIGURE 10
Stages in the development of certified reference materials (CRMs)



Notes: CRMs are tools that are considered major milestones of quality control of methods for the detection of trace contaminants such as phycotoxins (adapted from Hess, McCarron and Quilliam, 2007). Once the need has been established to produce a CRM, the feasibility must be explored; only after feasibility has been established can work begin on the production of the material.

The difficulty in predicting the occurrence of toxic algae and thus the sporadic occurrence of shellfish toxins also affects the monitoring necessary for exposure assessment. It is typically not sufficient to monitor shellfish toxins for a one-year period to assess their overall frequency of occurrence (see also Figures 5-8). In many cases, toxins may not occur for several years and then re-appear for one or several years. Therefore, regular surveillance over many years is required in order to assess the exposure of a population to a toxin group.

9.3 Risk characterization

Risk characterization investigates the nature and extent of the adverse health effects caused by a toxin group. In particular, risk characterization should examine whether only acute effects are encountered or whether also long-term effects exist. These considerations will result in the recommendation of an acute reference dose (ARfD) for protection against immediate effects, or of a total allowable or tolerable daily intake (TDI) for protection against chronic effects.

Wherever possible, risk characterization should be quantitative and should also outline options for risk management. For instance, such options will be given in the toxin-specific sections for covering different proportions of the population of shellfish consumers.

One of the most central questions of risk characterization is to establish the smallest dose of toxin consumed that will probably cause health problems. This level is called the lowest observable adverse effect level (LOAEL), expressed in micrograms or milligrams. As mentioned above, data acquired with validated methods are best for investigating which dose of toxin will make people sick. However, such data are not typically available at the early stages of risk analysis. This constellation is another example of the iterative nature of risk analysis. Indeed, the best data on poisoning events are acquired at an advanced stage when the toxin is already known to cause a problem and when quantitative methods are well advanced in their performance credibility. At this stage, government officials should already have organized monitoring of the toxin to prevent poisoning events, and, thus, paradoxically, the best data are obtained from failures of monitoring systems. Such failures sometimes occur when shellfish farms are newly established and the first crop consumed at inaugural events has not been analysed; this has been recorded both in Norway for OA-group toxins (Aune, 2001), and in Ireland for AZAs (McMahon and Silke, 1998). Other occasions for acquiring solid data for the estimation of the dose of toxin making people sick arise when a toxin group is known to occur in one country (A) but not in another (B). When the toxin appears in country B, poisoning may occur in the population of shellfish consumers and characterization of the concentrations causing the sickness may happen with methods applied or developed in country A. However, failures of monitoring systems also occur in situations where toxins are recognized as a problem but regulations are not followed closely. This is the case for most of the data obtained for the risk assessments described in the toxin-specific sections.

Once the LOAEL has been established, consideration is given to the nature of the risk, i.e. the effect and its gravity. From these considerations and from uncertainties associated with establishing the LOAEL, a safety factor is applied to estimate a no observable adverse effect level (NOAEL), again expressed in micrograms or milligrams. This NOAEL can also be expressed as a concentration in the human body, called the ARfD, expressed in micrograms per kilogram of bodyweight (typically NOAEL/60 for a 60 kg person).

The size of the safety factor depends very much on the data available to estimate the LOAEL. The first preference is always to derive the LOAEL from observations in humans. Where such data are available, then most typically a safety factor of ten is applied to account for variability between humans (different susceptibilities because of age, sex and genetic predispositions). In some cases, where large data sets are available on poisoning of many different people, including male and female, as well as children and aged consumers, it may be possible to reduce the safety factor to less than ten. However, this would also depend on the gravity of the effect at the LOAEL. If insufficient data were available from observations in humans, observations from animal experimentation would be considered to derive an NOAEL. In this case, the default safety factors would be 100 or 1 000, depending on the nature and gravity of the effect and other uncertainties.

Risk characterization should also quantitatively evaluate the risk as a function of the exposure data. This is particularly important for risk managers because it puts the gravity of risk in relation to the likelihood of occurrence. For instance, if long-term monitoring of a toxin indicated that concentrations in shellfish very rarely or never exceeded a certain level, and that no effects had been observed below this level, it would not be necessary to have a regulation in place for such a compound.

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