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REGULATIONS FOR MARINE MICROALGAL TOXINS: TOWARDS HARMONIZATION OF METHODS AND LIMITS

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Toxins produced by marine microalgae are harmful to humans and a serious threat to aquaculture and fisheries. Most seafood-producing countries have established monitoring programmes and regulations to protect public health from the risk of toxin exposure. However, there are disparities in current regulations regarding methods and applied limits for toxin control. Inconsistencies are especially evident for Diarrhetic Shellfish Poisoning (DSP) toxins. Epidemiological and toxicological data are necessary to assess risk, and to establish safe limits for the different groups of toxins. The scarcity or absence of pure toxins and certified reference materials has hampered toxicological studies and the development of suitable analytical methods. The World Trade Organization and the General Agreements on Tariffs and Trade encourage the harmonization of regulations on food safety requirements. The current policy on trade liberalization of seafood is presented, together with a review of the regulations for marine microalgal toxins. Activities on harmonization of methods and limits, particularly to DSP toxins, are discussed.

Marine toxins consist of a broad spectrum of biologically active substances, differing in origin, chemical structure, solubility and mechanism of action. They may be transferred to humans after consumption of seafood, including bivalve molluscs, gastropods, crustaceans and fish, and may cause a variety of gastrointestinal and neurological diseases. The main toxic syndromes of marine microalgal origin are Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Amnesic Shellfish Poisoning (ASP), Neurotoxic Shellfish Poisoning (NSP) and Ciguatera Fish Poisoning (CFP). Many seafood-producing countries have implemented monitoring programmes to protect public health from the risk of toxin exposure. The activities included in these programmes differ between countries, but typically include monitoring of oceanographic conditions (e.g. climate, currents and nutrients), toxic microalgal species and the toxin content of seafood. Toxicity is determined by a suite of testing procedures and the results are compared with established limits to prevent placement of unsafe products on the market. Some of these monitoring programmes have been agreed upon as official regulations, laying down the requirements for production areas and marketing, as well as the methods and limits to be used for sanitary control. However, current regulations have discrepancies in the methods and limits. Such disparities are especially evident in countries where there is free trade and unrestricted movement of goods and services. The lack of uniformity and, in some cases, the paucity of regulations, can lead to public health risks as well as unequal trade competition between countries. Future elimination of trade barriers, as proposed by the World Trade Organization, necessitates harmonization of these safety requirements.

This paper presents the international regulations pertaining to marine microalgal toxins, and current activities on harmonization of methods and regulatory limits are discussed.

WORLD TRADE ORGANIZATION AND GENERAL AGREEMENT ON TARIFF AND TRADE

The World Trade Organization (WTO) emerged as a consequence of historical efforts and negotiations intended to achieve the liberalization of international trade. The WTO includes the General Agreement on Tariff and Trade (GATT), which consists of about 125 countries that represent >90% of international trade. The basic policy of the organization is the progressive elimination of the barriers that protect domestic markets. The organization also provides an international forum to mediate disputes on trade policies.

Within this framework, sanitary standards or regulations may be used as non-tariff barriers. The GATT agreements on sanitary and phytosanitary measures acknowledge the right of WTO members to establish regulations for health protection, if they are based on scientific evidence and risk assessment, and do not discriminate among members where identical or similar conditions prevail. Sanitary regulations should not be applied in a manner that constitutes a disguised restriction to international trade. Therefore, when estab-

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lishing the suitable level of sanitary protection, each country is required to consider the aim of reducing as much as possible the negative effects on trade so that regulations do not involve a restriction higher than that required (Directive 94/800/EEC – Council of the European Communities 1994). Based on GATT, trade relationships should be carried out on the principles of harmonization, equivalency and transparency. This requires the establishment of common sanitary measures with adherence to international standards, the acceptance of different sanitary measures whenever the appropriate level of protection is demonstrated, and the elaboration of regulations with absolute transparency. WTO members should provide information on their sanitary regulations and notification of any changes to the regularities.

However, when examining current regulations pertaining to marine toxins, discrepancies between methods and limits are evident. GATT therefore encourages the uniformity of sanitary regulations in order to eliminate risks to consumers and to reduce obstacles to trade.

REGULATIONS FOR MARINE TOXINS

Regulations relating to marine toxins should be established on the basis of epidemiological and toxicological studies, as much as on the capability of testing procedures to identify and quantify toxin levels. In many cases, such studies have not been done and limits are set, despite the absence of toxicity data, by following the specifications of other countries. Establishment of appropriate regulatory levels requires robust toxicological studies that are time-consuming, costly and involve the use of large amounts of pure toxins that are in many cases unavailable. The detection limits of suitable testing methods are also poorly defined and the development and the validation of these methods have been hampered by the lack of pure toxins and certified reference materials (Van Egmond *et al*. 1992). In addition, the finding of new toxins and unknown toxicities, as well as the wide range of seafood that can act as vectors of toxin, make the establishment of common and rational guidelines and regulations more difficult.

A lack of uniformity is evident in the methods and limits applied for marine toxin control, even between countries for which free trade and unrestricted circulation of seafood already exist. In the European Union (EU), Directives 91/492/EEC and 97/61/EEC (Council of the European Communities 1991a and 1997 respectfully) set out the health conditions for the production and marketing of live bivalves intended for further processing or consumption. The Directive states the following regarding control of PSP, DSP and ASP:

(i) "The total Paralytic Shellfish Poison (PSP) content in the edible part of the molluscs (the whole body or any edible part seperately) must not exceed 80 $\mu g \cdot 100\ g^{-1}$ of mollusc flesh in accordance with the biological testing method – in association if necessary with a chemical method for detection of STX – or any other method recognized in accordance with the procedure laid down in aticle 12 of Directive 91/492/EEC. If the results are challenged, the reference method shall be the biological method" (p. L268/12).

(ii) The customary biological testing method must not give a positive result to the presence of DSP in the edible part of molluscs (the whole body or any edible part separately)" (p. L268/12).

(iii) The total ASP content in the edible part of the molluscs (the whole body or any edible part separately) must not exceed the level of 20 μg domoic $acid \cdot g^{-1}$ by an HPLC (high-performance liquid chromatography) method (p. L295/36).

The Directive is vague, and no specific biological methods have been established for PSP and DSP toxins. For DSP, the action limit is dependent on the detection limit of the biological method used. The need to eliminate discrepancies in methodology among EU member states and to harmonize European trade led to the establishment, by the European Commission, of the National Reference Laboratories on Marine Toxins and a Community Reference Laboratory. These facilities were to set up and coordinate a network for the exchange of information, knowledge and experience, and to create a forum to establish agreement on toxicological and methodological issues (Fernández *et al*. 1996).

Agreement on various methods and limits has been achieved by the network of EU Reference Laboratories within the last few years. In some cases this has led to amendments in EU legislation, whereas others will form the basis of future regulations. Working groups, consisting of experts and members of the EU laboratories, have also been created to investigate other issues.

A similar programme addressing harmonization has been implemented among the 18 countries belonging to the Asia Pacific Economic Cooperation (APEC) organization. One of the goals of APEC is to foster free trade within member economies, with respect to their ability to manage fisheries resources. An ambitious "Red Tide Toxic Algae Project" has been established in an attempt to achieve common seafood safety standards and legislation (APEC 1997).

Paralytic Shellfish Poisoning

Among all the seafood poisonings, PSP is one of the most severe threats to public health, affecting most coastal areas in the world. Saxitoxin (STX) and its analogues are produced mainly by a number of dinoflagellates belonging to the genera *Alexandrium, Gymnodinium* and *Pyrodinium* (Yasumoto and Murata 1993). Although bivalve molluscs are the most common vectors of PSP, gastropods, crustaceans and pelagic fish have also been reported as vectors of the toxins (Shumway 1995). Many affected countries have established regulations, including stipulated methods and action limits.

The method used most often to determine PSP toxins is the mouse bioassay (AOAC 1990), which has been validated and standardized by the Association of Official Analytical Chemists (AOAC). This reference method is the only procedure recognized internationally for quantifying PSP toxicity. Although some variation is observed in the acceptable regulatory level and in the units used for expression of the toxicity (40–80 μg PSP·100 g^{-1} tissue or 200–400 mouse units (MU)·100 g^{-1} tissue – Van Egmond *et al*. 1992), most countries have agreed on the value of 80 μg STX equiv.·100 g^{-1} tissue.

The PSP mouse bioassay involves acidic aqueous extraction of the tissue, followed by intraperitoneal injection of the extract into each of three standardized mice. The mice are observed for classical PSP symptoms such as jumping in the early stages, followed by death within 15 minutes as a result of respiratory arrest. The time from initial injection to mouse death is recorded and the toxicity is determined in mouse units from Sommer's table (AOAC 1990). One mouse unit is defined as the amount of toxin required to kill a 20 g mouse within 15 minutes. After standardization with STX, mouse units are converted to toxicity units (μg of STX equiv·100 g^{-1} tissue). The assay sensitivity depends very much on the strain of the mouse, and for this reason standardization of the bioassay is crucial (Cembella *et al*. 1995). As STX is defined as a chemical weapon, it has become inaccessible in many countries, especially in those that have not signed or ratified the Convention for the Prohibition of Chemical Weapons. Restrictions in transfers and re-transfers of STX between countries have materialized as a consequence of that Convention, which may ultimately hinder the standardization of the assay in some countries.

Although the method has been extensively validated, there are some aspects of the procedure that need clarification in order to improve the reproducibility of the assay. These include the extraction conditions, the solvent used to dilute extracts, the pH of the extract and the application of the analysis to canned shellfish and other processed products.

For PSP, members of the EU National Reference Laboratories on marine toxins agreed that the biological method referred to in Directive 91/492/EEC should be that described in the "AOAC Official Methods of Analysis" (AOAC 1990) and that the tolerance level should be that specified in the Directive. The group recognizes the importance of the pH of the extract and recommends that the range in pH values be kept as narrow as possible to reduce variability and improve reproducibility. The effectiveness of procedures such as evisceration and canning in reducing PSP toxicity has led to some countries establishing different regulatory limits, depending on the form of consumption of the shellfish. Therefore, in the United States and Canada, shellfish destined for canning or subjected to an evisceration step might be harvested with PSP toxin concentrations >80 μg STX equiv.·100 g^{-1} tissue (Cembella and Todd 1993, NSSP 1990). In the case of EU legislation, the application of detoxification procedures is limited by Directive 91/492/EEC, which prohibits harvesting toxic shellfish. There is only one exception, which applies to the canning of the bivalve *Acantochardia tuberculata*. The exception allows Spain to authorize harvesting when PSP levels in the edible parts exceed 80 μg of STX equiv.·100g^{-1} tissue, but <300 μg of STX equiv.·100 g^{-1} tissue (Directive 96/77/EEC – Council of the European Communities 1996). However, the bivalves must undergo a prescribed heat treatment, and the final product must not contain a PSP toxicity level detectable by the mouse bioassay, and each consignment must be tested (Burdaspal *et al*. 1998).

Regarding PSP testing procedures in future, ethical and technical considerations encourage the development and application of *in vitro* assays that might complement or replace the mouse bioassay. Concerning public health protection, functional assays based on the biological action of the toxins are preferred. Neuroblastoma assays (Kogure *et al*. 1989, Gallacher and Birkbeck 1992, Jellet *et al*. 1992) seem to be among the most promising, and after refinement and extensive validation might replace the mouse bioassay. Receptor binding assays (Davio and Fontelo 1984, Vieytes *et al*. 1993) may also be good techniques, if radio-labelled compounds can be replaced by chemiluminiscent or fluorescent compounds.

Diarrhetic Shellfish Poisoning

Of all the groups of marine toxins, those included in the DSP group are the subject of greatest controversy.

There is no general consensus as to which liposoluble toxins should be regarded as DSP toxins, which toxins should be monitored and regulated, the acceptable levels of toxin, or the most appropriate testing procedures. Three groups of toxins have been historically included in the DSP group: okadaic acid (OA) and its analogues, the pectenotoxins (PTXs) and yessotoxins (YTXs – Yasumoto and Murata 1993). There is general agreement as to the inclusion of the OA group, but there are conflicting opinions on the inclusion of the other two groups.

DSP toxins share polyether cyclic structure and solubility properties. Generic extraction procedures and conventional mouse bioassays do not discriminate between them. However, their biological activity and toxicological properties are considerably different. OA and its analogues cause diarrhoea (Murata *et al*. 1982), they inhibit protein-phosphatase enzymes PP1 and PP2A (Bialojan and Takai 1988) and are also potent tumor promoters (Fujiki *et al*. 1988). They are produced by different *Dinophysis* and *Prorocentrum* species and have been responsible for DSP intoxication in many areas of the world, but mainly in Europe and Japan.

PTXs, a group of neutral polyether lactones, do not cause diarrhoea in mice when injected intraperitoneally, but do when administered orally. Serious damage has also been observed in the intestine and the liver of mice. They do not inhibit protein phophatase enzymes but show potent cytotoxicity (Terao *et al*. 1990, 1993). PTX2 is produced by *Dinophysis fortii* and the other PTXs are products of metabolic oxidation in the digestive glands of shellfish (Yasumoto *et al*. 1989). PTXs have been identified in phytoplankton and/or shellfish species from Japan, Norway and Italy (Lee *et al*. 1988, Yasumoto *et al*. 1989, Draisci *et al*. 1996).

The third group of DSP toxins, the YTXs, includes sulfphated polyether compounds of similar structure to brevetoxins. They do not cause diarrhoea and do not inhibit protein phosphatase enzymes. Mouse intraperitoneal toxicity is high (100 $\mu g \cdot kg^{-1}$) and animal studies showed severe damage to the heart muscle when YTX was given intraperitoneally (Terao *et al*. 1990, 1993). However, oral toxicity is much lower (>1000 $\mu g \cdot kg^{-1}$) than that of OA or the PTXs (Ogino *et al*. 1997). The production of YTXs by the marine dinoflagellate *Protoceratium reticulatum* has been confirmed (Satake *et al*. 1997). Homo-YTX has been detected in a net sample dominated by *Lingulodinium polyedrum* from the Adriatic Sea (Tubaro *et al*. 1998), but its presence has not been confirmed in cultured cells from the same species isolated in the United States, Canada, Japan and New Zealand. YTXs have been found in shellfish in Japan, New Zealand, Chile, Italy and Norway (Yasumoto and Murata 1993, Zhao *et al*., 1993, Ciminiello *et al*. 1997). PTXs and YTXs are believed to be distributed worldwide, because the phytoplankton species producing these toxins are common in many coastal areas.

In addition to these lipid-soluble toxins, a new diarrhetic toxin has been isolated from shellfish on the coast of Ireland, although the source remains unknown (Satake *et al*. 1998a, b). Human symptoms include nausea, vomiting, diarrhoea and abdominal cramps. The new toxin, named azaspiracid, occurs seasonally and exhibits a highly oxygenated polyether structure, indicating that a dinoflagellate is the likely source. The new toxin can be detected by the rat or mouse bioassay, with the symptoms in mice similar to those produced by intraperitorial injection of PSP toxins.

Concerning the sanitary control of DSP toxins, there are disparities in the methods and in the criteria for a positive result. Mammalian bioassays are widely applied for DSP toxicity determination. However, there are considerable differences in the procedures. Assay selectivity, specificity and toxin recovery depends upon the selection, and ratio of the organic solvents used for extraction. Of all the DSP mouse bioassay procedures, the one developed by Yasumoto *et al*. (1978), in which an acetone extraction is specified, detects the broadest range of toxins. Liquid-liquid partitioning steps carried out with different organic solvents (Yasumoto *et al.* 1984, Le Baut *et al*. 1990) improve the specificity of the bioassay, but may lead to a loss of toxins. Bioassay procedures as diverse as the oral dosage rat bioassay (Kat 1983), used in some EU countries, and the intraperitorial injection of mice assay are not equivalent. This is because the former technique quantifies only the diarrhetic effect of certain DSP toxins, whereas the latter assay yields an estimate of global DSP toxicity (Cembella *et al*. 1995). Regarding regulatory levels, most countries have set the limit at the detection limit of the analytical method used. Japan was the first country to establish a limit (5 $MU \cdot 100\ g^{-1}$ tissue), based on an epidemiological study (Yasumoto *et al*. 1978). This limit has also been adopted in Norway, Korea and New Zealand.

Directive 91/492/EEC establishes that the customary biological method must not give a positive result for the presence of DSP toxins, but it does not clarify the interpretation of a positive result and it also does not specify which biological method should be used. In practice, most countries that apply mouse bioassays use the survival time for the determination of DSP toxicity, but without consensus on the appropriate observation period. The acceptable criteria vary from two of three mouse deaths in <5 h to <24 h. Depending on the ratio of hepatopancreas to the whole body, a

survival time of five hours may be insufficient to assure product safety, because concentrations of DSP in the whole body may be sufficiently high to produce diarrhoea (Míguez *et al*. 1998). The risk may also increase if, in addition to OA, DTX2 or acyl-derivatives of DSP toxins are present in the shellfish.

Members of the EU National Reference Laboratories recently agreed that monitoring for DSP should include all the acetone-soluble toxins, including those with no diarrhetic activity, until further toxicological data are available to establish new limits. From a human health perspective, methods detecting a broad range of toxins are preferred. The mouse bioassay established by Yasumoto *et al*. (1978), with a survival time of 24 h as the criterion for a positive result, was chosen as the assay providing the highest level of sanitary protection. It was recognized that this method lacks specificity, but that this disadvantage could be overcome by the use of complementary analytical strategies that need not necessarily be biological (Anon. 1996). Turkey, Venezuela, Chile, Uruguay and Thailand regulate DSP on the basis of the Yasumoto *et al*. (1978) mouse bioassay. The United States and Canada have no official regulations on DSP, although Canada may issue an informal advisory warning, following positive results by mouse assay and/or the fluorescence HPLC method.

The term "diarrhetic" is not fully justified for all the DSP toxins and re-evaluation of their toxicity is likely to lead to some of the toxins being removed from the classification of DSP toxins (Van Apeldoorn *et al*. 1998). Preliminary studies suggest that the health risks from YTXs may be much lower than those of OA and PTXs, and that different limits could be set for each group. However, for the purposes of control, it is difficult to monitor the different groups of "DSP-associated" toxins separately. A fluorimetric HPLC method for YTX detection that may also be useful for detecting certain PTXs has been developed (Yasumoto and Takizawa 1997), but pure toxins and certified reference materials are not available for its routine implementation. HPLC methods provide useful information on toxin profiles, but they do not detect conjugated forms and standards are not available for all the OA analogues. This deficiency makes it difficult to apply such methods for sanitary control. Enzyme inhibition assays with colorimetric or fluorescence detection (Tubaro *et al*. 1996, Vieytes *et al*. 1997) have been recognized as highly promising and after refinements and validation might be as effective as the above-mentioned procedures for detection of OA and its analogues. However, acyl-derivatives of OA and other analogues require a previous hydrolysis in order to be detected by the latter procedures.

Amnesic Shellfish Poisoning (ASP)

ASP is produced by the ingestion of seafood contaminated with domoic acid (DA), an excitatory amino acid produced by some species of the pennate diatom of the genus *Pseudonitzschia*. The first toxic outbreak was in Canada in 1987. Subsequent toxic episodes have been reported in other countries, but have not included human poisonings.

Countries that have established regulations for ASP include the United States, Canada, New Zealand (Wright *et al*. 1989, NSSP 1990 Trusewich *et al*. 1996) and the EU countries. The limit of ASP toxin is 20 $mg \cdot kg^{-1}$ edible meat. The United States Food and Drug Administration has established a quarantine level of 30 ppm of domoic acid in cooked viscera from Dungeness crabs (Shumway 1995).

HPLC methods have been established as appropriate testing procedures for ASP detection. The method with UV detection has been validated in an AOAC collaborative study by Lawrence *et al*. (1989) and is used in many countries as a screening procedure. A common acidic extraction for both PSP and ASP toxins is performed in the method. Depending on the shellfish species, low ASP toxin recoveries may result from the AOAC procedure for PSP toxins, so other extraction procedures, such as that developed by Quilliam *et al*. (1995), have been widely used when analytical accuracy is necessary. As with PSP toxins, not only bivalve molluscs, but also crustaceans and herbivorous fish such as anchovy have been reported as vectors of ASP toxins (Shumway 1995), and therefore the latter should be included in surveillance programmes.

Domoic acid isomers, exhibiting biological activity, have been found in shellfish and plankton, but in small quantities. Most isomers are less toxic than domoic acid, which is regarded as the principal toxin responsible for ASP. As for the other groups of toxins, the development of functional assays for domoic acid is highly desirable. A competitive receptor-binding assay has been developed for the detection of domoic acid and its analogues (Van Dolah *et al*. 1994). The primary disadvantage of this technique for application in routine screening is the use of radio-labelled compounds.

Neurotoxic Shellfish Poisoning

NSP is a seafood intoxication produced after the ingestion of shellfish contaminated with brevetoxins, produced by the dinoflagellate *Gymnodinium breve*. The United States and New Zealand are currently the

areas affected directly by NSP (McFarren *et al*. 1965, Ishida *et al*. 1995). The testing procedure in use in the United States is the mouse bioassay (APHA 1985), and the acceptable level is 20 MU·100g^{-1} tissue. The same level is employed in New Zealand (Trusewich *et al*. 1996), using a mouse bioassay equivalent to the APHA method (Hannah *et al*. 1995). This level corresponds to the lower sensitivity limit for 6 h continuous observation. The EU Directive 91/492/EEC makes no mention of NSP toxins, which have not been reported in European coastal waters.

Ciguatera Fish Poisoning

CFP is a complex poisoning syndrome produced after the consumption of certain species of tropical and subtropical reef fish. Tropical Pacific and Caribbean regions, including Australia and French Polynesia, are the primary areas affected by it. Hawaii, Puerto Rico and Florida are also affected. The analogues of the principal toxins, ciguatoxins are produced by an epiphytic dinoflagellate *Gambierdiscus toxicus*, but they are subject to considerable biotransformation in vector species (e.g. fish). The presence of ciguatoxins in fish can be detected by means of mouse bioassays, involving laborious and time-consuming purification procedures. A suite of chemical and *in vitro* biochemical and biological assays have been developed for ciguatoxin detection (Yasumoto and Murata 1993). However, for sanitary control of ciguatoxins, sampling is particularly difficult. There are no effective testing programmes for CFP, and the most widespread sanitary measure applied for its prevention is the prohibition of the sale of fish species known to be potentially toxic, or for which some CFP outbreaks have been reported. Directive 91/493/EEC (Council of the European Communities 1991b) prohibits the placing of fish containing ciguatoxins on the market.

EXAMPLES OF PROCEDURES FOR INTERNATIONAL SEAFOOD TRADE

With regard to international seafood trade with the major trading blocks, the following procedures apply:

(i) In the EU, the European Commission Decision 98/571/EC lists the harmonized "third countries". These are countries outside the EU that fulfil the equivalent conditions for the production and placing on the market of bivalve molluscs, echinoderms, tunicates and marine gastropods, laid down by Directive 91/492/EEC. The import of such seafood from these countries into the EU is permitted after veterinary control at border inspection posts. When a sanitary problem is detected from a country outside the EU, an EU Decision may be made to prohibit importation.

(ii) In the United States, the Food and Drug Administration (FDA) uses the updated National Shellfish Sanitation Program (NSSP) manual for certifying foreign shellfish sanitation programmes. To accomplish this, the FDA seeks to establish international Memoranda of Understanding (MOUs) with foreign countries that wish to export shellfish to the United States. Once a country has an effective MOU, the shellfish control authority submits documentation of their certified shellfish dealers to the FDA.

Canada follows a similar procedure. All bivalve mollusc shellfish that are processed for export or those that are imported must meet the requirements of the Fish Inspection Regulation (FIR). For shellfish, the sanitary requirements are set out in the Canadian Shellfish Sanitation Program (CSSP). Under the FIR, the importation of fresh or frozen raw bivalve molluscs is restricted to specific countries. The CSSP has been reviewed in the past by the United States FDA to determine the compliance of the CSSP with NSSP requirements, as provided by the Bilateral Memorandum of Agreement between that country and Canada. The CSSP is equivalent to, but not identical to, the NSSP.

THE FUTURE

The establishment of rational and harmonized regulations for marine toxin control requires further effort in the following areas:

(i) production of pure toxin standards and certified reference material;
(ii) toxicological studies for those groups of toxins where information is insufficient to allow for risk assessment; and
(iii) development and validation of *in vitro* assays and/or alternative chemical analytical methods. Interaction between international networks and organizations dealing with marine toxin regulations is highly desirable.

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