3.5.5 ToR f) Review state-of-the-art and ongoing research and managerial activities related to emerging benthic HABs in ICES countries

Beatriz Reguera, IEO, Vigo, Spain

1. BACKGROUND

HAB events caused by benthic dinoflagellates of the genera *Gambierdiscus* (Ciguatera Fish Poisoning) and *Ostreopsis* (palytoxins) have been known for long in tropical and subtropical regions of the Atlantic, Pacific and Indian oceans. Nevertheless, problems associated with species of these genera have emerged in European countries in the 2000's raising concern about their potential relation with climate change. Health authorities and food safety agencies from temperate latitudes were not prepared to control the occurrence of these benthic HAB's toxins in seafood and marine aerosols, or even to recognize the symptoms in affected consumers. This concern has contributed to the establishment in 2010 of a new SCOR-IOC GEOHAB *Core Research Project on HABs in Benthic Systems* (GEOHAB 2012; Zingone et al. 2012) and triggered the celebration in April 2011 of a conference dedicated to *Ostreopsis* (ICOD = *International Conference on Ostreopsis Developments*) during the meeting of the French Phycological Society (SPF) in Villefranche-sur-Mer (Lemée et al. 2012).

Ciguatera in a broad sense may include a complex mixture of at least three groups of lipophilic toxins produced by more than three different genera (*Gambierdiscus*, *Ostreopsis*, *Prorocentrum, Amphidinium*) of benthic dinoflagellates co-occuring in the same tropical-subtropical habitat (Morton et al. 1992, 1997). Nowadays we know that, concerning benthic HABs, ciguatera toxins in a strict sense (ciguatoxins, maitotoxins) are produced only by species of the genus *Gambierdiscus*; palytoxins and its analogs by *Ostreopsis* spp.; and okadaic acid and its congeners (diarrhetic shellfish toxins) by benthic species of *Prorocentrum*.

1.1 Ciguatera Fish Poisning (CFP)

Ciguatera is a seafood poisoning caused by eating tropical coral reef fish that act as carriers of the ciguatera toxins (CTX) (Yasumoto, 2005). Ciguatera Fish Poisoning (CFP) is endemic in tropical areas within the Atlantic Ocean (in particular in the Caribbean Sea), the Indian Ocean, and in the Pacific (in particular in Polynesian islands) (Lewis, 2001). The term "ciguatera" comes from "cigua", the name given in Cuba to a carnivorous snail (*Livona picta*) identified by popular wisdom as an important source of ciguatera symptoms. Although descriptions of fish intoxications presumably caused by ciguatoxins exist since pre-Christian times, the first detailed report of CFP dates back from 1774 and was provided by a surgeon's mate of Captain Cook's South Pacific exploration aboard the HMS *Resolution* when the crew was intoxicated after eating fish in New Caledonia (Lehane, 1999).

CFP is by far the most extended non-bacterial seafood borne intoxication (Lehane and Lewis, 2000) and mainly affects coastal populations from developing countries where artisanal fisheries constitute an important part of their staple food. Toxins are produced by benthic microalgae which live attached to seaweeds or other substratum and are transmitted to humans through herbivorous and carnivorous fish. At the moment, there is not a realistic, cost-effective way to monitor CTXs in seafood and the only possible prevention is to avoid consumption of risk species from risk areas. More than 50,000 cases are reported every year but it is assumed that less than 10% of them are communicated to the health authorities. Symptoms of the intoxication are used to diagnose and distinguish CFP from other seafood intoxications. However, the confirmation of cases of CFP relies upon the detection of CTXs in the remaining meal

or within the plasma of patients (Bottein, 2007). Therefore, it is important to have adequate CTX quantification methods to diagnose CFP cases and moreover, to prevent intoxications through the analysis of consumable fish (see review of methods in Caillaud et al., 2010). In tropical Pacific regions, such as the French Polynesia, where research and management of ciguatera started since the 60's, the ciguatera risk assessment programme is based on two main activities (i) epidemiological survey of marine biotoxin intoxications throughout the islands and (ii) the microalgal toxin-based field monitoring in the lagoons, complemented with awareness education of the local population. In this way, identification of the risk species but above all of the risk areas (probably related with development of more toxic strains) have been identified (Darius et al., 2007).

In a risk assessment study carried out in Puerto Rico between 1997 and 1998, Tosteson (IOCARIBE-ANCA, 1998) estimated an incidence of 18000 cases per year of CFP in a population of 3.5 million (i.e. 51 cases per 10,000 population per year). This author calculated a loss of 8 to 10 million dollars per year due to: i) loss in revenue to the fishers and distributors when there is an outbreak of ciguatera and potential consumers at least momentarily cease to purchase fish; ii) medical costs incurred by those persons poisoned and iii) the resulting loss of income due to the interruption of employment among poisoned consumers. According to Tester et al. (2009, 2010), between 1996 and 2006, highest rates of CFP in the Caribbean were 34 and 59 per 10,000 population per year in Antigua-Barbuda and Montserrat (Lesser Antillles) and these rates represented an increased incidence of CFP as compared with reports from the 1980's. In Canada, Todd (1997) estimated there could be as many as 300 cases of ciguatera poisoning each year, either from tourists visiting tropical areas or from imported tropical fish consumed in Canada. Assuming that each of these cases costs $4,000, the total annual cost could be as high as $ 1,236,000.

Interest for CFP in Europe increased in parallel with increased tourist activities in tropical regions and reports from European hospitals (from France, Spain, the Netherlands, Germany, and Italy) about citizens who suffered CFP symptoms during their holidays or upon their return from the tropics. Nevertheless, reports in recent years indicated that CFP was present in areas of Africa close to Europe and in the Macaronesia islands (Azores, Canary, Cape Verde and Madeira Islands). In 2004, CFP was first confirmed after consumption of flesh from fish caught in the Canary Islands (Pérez-Arellano et al., 2005), the presence of CTXs was also suspected in fish from Madeira in 2007 and 2008 (Gouveia et al., 2009), and for the first time a new toxic species of this genus, *Gambierdiscus eccentricus*, was reported from the Canary Islands in 2004 (Fraga et al., 2011). In all these cases, affected consumers had eaten large (up to 60 kg) Carangidae fish of the genus *Seriola*. In addition, dinoflagellates of the genus *Gambierdiscus* have been recorded in the Mediterranean Sea since 2003 (Aligizaki and Nikolaidis, 2008). All these reports from the 2000's have raised the concern about a possible spreading of CFP causing *Gambierdiscus* species to subtropical regions from which they had never been reported before.

In parallel, an extensive revision of the six already described species of *Gambierdiscus*, based upon morphological and phylogenic analysis, led to the description of four new species, *i.e. G. caribaeus*, *G. carolinianus*, *G. carpenteri*, and *G. ruetzleri*, in the Caribbean and southern Atlantic US area (Litaker *et al.*, 2009). It was also proposed that the original species, *G. toxicus* (Adachi and Fukuyo, 1979) may in fact include multiple species.

Incidences of CFP, according to the most recent mapping of this syndrome, are now distributed between parallels 35ºN and 35ºS. A problem identified by the GEOHAB CRP was the heterogeneity of macroalgal substrata where benthic HABs occur and hence, the difficulties to obtain comparable data of dinoflagellates abundance between regions. This led this group of experts to recommend a standard artificial substrata to be deployed and recollected in the study areas (GEOHAB 2012).

1.2 *Ostreopsis* events

Dinoflagellates of the genus *Ostreopsis* have been cited in the western Mediterranean coast in the last century (Meunier, 1919; Halim, 1960), but it was not until the early 2000's when harmful events, causing dermatitis and respiratory problems, were reported from the Mediterranean Seas in Italian, Spanish, Greek, French, Tunisian and Algerian coastal areas and linked for the first time with proliferations of *Ostreopsis* (*O*. cf *ovata* and *O*. cf *siamensis*) (Illoul et al., 2012; Mangialajo et al., 2012; Sansoni et al., 2003; Vila et al., 2008). Phylogeographical considerations on the genus *Ostreopsis* showed that *O.* cf. *ovata* is widely dispersed throughout tropical and warm temperate coastal areas and that in the North Atlantic/Mediterranean region it represents a panmictic population that is highly divergent from Indo-Pacific populations (Penna et al., 2012)

Six *Ostreopsis* species have been reported as responsible for toxic events, of which three have been shown to produce potent toxin analogues of palytoxin, which are complex, high molecular weight, and water-soluble polyalcohols (Riobó and Franco, 2011). After the discovery of ovatoxin-*a* in Mediterranean strains of *O.* cf. *ovata* (Ciminiello et al., 2008), five more ovatoxin types and two mascarenotoxins were detected for this species (revised in GEOHAB, 2012). Strains of *Ostreopsis* cf. *ovata* isolated from the French Mediterranean coast were identified with molecular methods and their toxins analysed by LC-MS. They contained ovatoxin-a (OVTX-a) (up to 55 $pg.cell^{-1}$) as their major toxin component and small amounts of palytoxin (PLTX) (max. of 2.5 $pg.cell^{-1}$) (Sechet et al. 2012).

Palytoxin like substances were found in plankton samples collected in summers 2005 and 2006 along the coasts of the Ligurian Sea when up to 200 people were hospitalizad presenting cough, dyspnoea, sore throat, rhinorrhea, fever, headache, lacrimation, nausea/vomiting and dermatitis (Ciminello et al., 2006; Durando et al., 2007). Interestingly, other intense *Ostreopsis* blooms have occurred in the same and other Mediterranean areas in summer, without any report of similar syndromes (Durando et al., 2007; Tubaro et al., 2011).

So far, demonstrations of the presence of PLTs in the aerosol during *Ostreopsis* blooms, or that the symptoms occasionally reported were actually caused by PLTs are weak (Tubaro et al., 2011). It is a priority to investigate the complex biological and environmental interactions . that modulate the toxic outbreaks during the co-occurrence of intense blooms in calm waters followed by persistent onshore winds (GEOHAB, 2012). Further, it can not be discarded that other substances different from PLT are the actual bioactive compounds in the toxic aerosols.

Aligizaki et al. (2008) reported the presence of p-PLT contamination of shellfish (33.3 to 97.0 mg p-PLT · kg^{-1} tissue) by natural populations of *Ostreopsis* in the Eastern Mediterranean Sea. This represents, so far, the only report on the presence of putative palytoxin-like compounds (p-PLT) in shellfish.

The deleterious effects of *Ostreopsis* cf. *ovata* blooms on the meiofauna (metazoans 40 μm to 1 mm in size) inhabiting the very common brown macroalga *Halopteris scoparia* were studied in shallow coastal lagoons along the French and Italian Mediterranean coasts. Changes in the community structure were associated with high abundances of *Ostreopsis;* the most affected organisms were the *nauplii* suggesting a negative impact on harpacticoid copepod reproduction (Guidi-Guilvard et al., 2012). Invertebrate mass mortalities linked to *O. ovata* blooms have to be iinterpreted with caution, because other causes, such as oxygen depletion or high seawater temperature have to be considered. Faimali et al. (in press) carried out an ecotoxicological screening to investigate the toxic effects of different concentrations of *O. ovata* (cultured in the laboratory and sampled in the field during blooms) on crustaceans and fish larvae. They found that *Artemia salina* was the most sensitive species even at concentrations below the "Environmental Alarm Threshold" set by the Italian Ministry of Health. 2011

1.3 European Union Regulations

European Union regulations state that " *Fishery products containing biotoxins such as ciguatoxins or other toxins dangerous to human health*"(EU, 2008), and regulations EC 853/2004 (EU, 1986) and EC No 1021/2008 (EU, 2008) control imports of fish species from genera well known to be associated with different kinds of ichtyosarcotoxisms ("*fishery products derived from poisonous fish of the following families are not placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae*"). European regulations give neither indications about a reference analysis method for CTXs, nor about regulatory safety limits in fisheries products. This lack of specification should not be interpreted as a lack of interest, but as a reflection of the difficulties that laboratories dealing with ciguatera have encountered during the last decades regarding the definition and standardization of CTX analytical methods and establishment of safety limits. Even in laboratories with advanced LC-MS equipments the lack of CTXs standards hinders the possibility of analysing complex matrices with a growing number of described congeners. The European Food Safety Authority (EFSA) is currently completing a mandate to issue a scientific opinion on ciguatoxins (Mandate M2006-0060 on Emerging toxins-ciguatoxins) in response to a question raised by the European Commission (DG-SANCO). National regulations specifically addressing CFP do exist in some EU countries with overseas territories in ciguatera endemic areas. For example, in French Polynesia, groupers, snappers, barracuda and surgeonfish are banned for sale (Bagnis, 1992).

2. ONGOING RESEACRH AND MANAGEMENT OF BENTHIC HABS IN ICES COUNTRIES

2.1 Research and Management of Benthic HABs in Spain

2.1.1 Ostreopsis blooms research and management in northern in Catalonia

(Magda Vila, Marine Biology and Oceanography Department, ICM-CSIC, Barcelona. magda@icm.csic.es)

Ostreopsis blooms have been studied intensively during two years (2009-2010) in Sant Andreu de Llavaneres beach (NW Mediterranean Sea), a hot spot for toxic aerosol events in the Catalan coast, in the framework of the EBITOX project. *Ostreopsis* abundances in seawater and epiphytic on macrophytes have been monitored monthly during winter and weekly or biweekly during the blooming period, and quantified by

light microscopy (Vila et al., in prep.). During the two years of study palytoxin-like compounds (PLTX) have been analysed in the microepiphytic assemblages (Riobó et al., in prep.). At specific times the quantifications have also been done by qPCR assay (Casabianca et al., in press).

It is suspected that bacterial populations associated to *Ostreopsis* in the natural assemblages could contribute to the bloom toxicity. Thus, bacterioplankton and epiphytic bacteria assemblages have been characterized during the two sampling years of the project.

Ostreopsis showed a marked seasonality, being detected from July to mid-November. Molecular tools (PCR) revealed that the bloom was clearly dominated by *O*. cf. *ovata*, although *O*. cf. *siamensis* was detected in some occasions. The epiphytic *O*. cf. *ovata* bloom maintained concentrations above 10^5 cells·g^{-1} w.w. during the hot season, forming a brown mucilage that coated the benthic community of macrophyte. Usual *Ostreopsis* abundances in the water column during the bloom season ranged between 10^3 to 10^4 cells·L^{-1}; however, numbers above 10^5 cells·L^{-1} were sporadically recorded.

High Volume Air Pump samplers were installed in order to monitor the marine aerosol in the beach. The presence of *Ostreopsis* in the aerosol filters was investigated using both Scanning Electron Microscopy and molecular tools (PCR); the potential presence of PLTX was tested by LC-FLD and haemolytic assay. In the aerosols, SEM observations revealed the presence of some marine microalgae (mainly diatoms), whereas the presence of *Ostreopsis* was unclear using SEM; however, molecular assays revealed the presence of *O*. cf. *ovata*. In particular, qPCR assays estimated *O.* cf. *ovata* abundances up to 100 cells per filter during an outbreak in summer 2010 (Casabianca et al., in press). These concentrations are below the detection limit of the existing PLT quantification tools. In contrast, PLT ranged between 0.1 and 1.2 pg/cell in the microepiphytic assemblages.

Data to determine the relationship between *Ostreopsis* concentrations, health disturbing symptoms and meteorology are currently been analysed. The characterization of the bacterioplankton revealed that the highest abundances of planktonic bacteria coincided with the *Ostreopsis* bloom period. In turn, epiphytic bacteria assemblages (on macrophytes) were significantly more active than during periods when *Ostreopsis* was not present. In addition, epiphytic bacteria were significantly more diverse and more active than bacteria in seawater. DGGE gel-based sequential analyses detected the presence of several bacterial strains that could contain tetrodotoxin (e.g. *Vibrio*) (Borrull et al., in prep.). Following this relevant observation, huge amounts of epiphytic bacteria have been concentrated and processed to analyse the potential presence of certain toxins which may contribute to the toxicity of the *Ostreopsis* blooms. Analyses are in progress.

The aforementioned study has been financed by the Spanish national project EBITOX: "*Study of biological and toxinological characteristics of benthic dinoflagellates posing a risk to human health*" (CTQ2008-06754-C04-04). PI: J.M. Franco (Unidad Asociada IIM-CSIC and IEO-Vigo). A. Penna (Biomolecular Department, Urbino University, Italy) and M.M. Sala (Marine Biology and Oceanography Department, ICM-CSIC, Barcelona) have participated as external collaborators of this project.

Management: As part of the cooperation between the EBITOX research project and the water quality monitoring program, ICM-CSIC scientists involved in the project contact the ACA (Catalan Water Agency) whenever *Ostreopsis* concentration reaches ~10^5 cells·g^{-1} w.w. macroalgae or ~10^4 cells·L^{-1} in seawater at Llavaneres. This thresh-

old is based on empirical results suggesting that at those concentrations health problems in humans can occur if the wind is blowing landwards (Vila et al. 2012).

2.1.2 Benthic HABs detection and research in southern Catalonia

(Pablo de la Iglesia, IRTA, Tarragona. Pablo.delaIglesia@irta.cat)

Research conducted to address emerging toxins and/or benthic HABs have been developed within the framework of several projects. The exposure to regulated and non-regulated (emerging) toxins have been assessed by a national project in samples from the shellfish harvesting areas and also from retail markets and expedition centres (Project INIA, RTA2009-00127-00-00). Benthic HABs, such as *Ostreopsis* spp. in the Ebro Delta surrounding area (NW Mediterranean Sea) have been also the subject of study in another project. Population dynamics in the environment as well as laboratory experiments with some microalgae isolates are still in progress, and some conclusions are expected regarding the impact of benthic blooms on sea urchins populations, aquaculture and human health (project JACUMAR). Emerging toxins with special focus on food safety and human health will be covered in a EU-context through the 7th framework KBBE.2012.2.4-01 project "ECsafeSEAFOOD" (Grant agreement No 311820), together with other seafood contaminants. Other related activities dealing with HABs and microalgae are the exploitation of remote sensing and modelling as early warning tools against HABs by the "Purga de Mar" project, (INNPACTO, IPT-2011-1707-310000), and the use of diatoms as nano-structured supports in the design of novel biotechnologies (DIANA project, MINECO, BIO2011-26311)

2.1.3 Ostreopsis distribution in the Atlanctic coast of Iberia

(Enma Orive, Plant Biology and Ecology Department, University of the Basque Country, emma.orive@ehu.es)

The distribution of *Ostreopsis* cf. *siamensis* and *Ostreopsis* cf. *ovata* in the Atlantic coasts of Iberia have been studied by a group in the University of the Basque Country (David et al., 2012). They found that temperature ranges observed in the study area could not explain the species distribution but presumably the length of the warm period was the key factor. Their hypothesis is that for *Ostreopsis* to be present in a certain area three continuous months with SST above 19.5°C may be necessary. The morphological and phylogenetical characterization of *Ostreopsis* species in the same region has been studied by the same group (David et al., accepted).

2.1.4 Ciguatera and other Benthic HABs in the Canary islands

(Carolina Pérez, Banco Español de Algas BEA, Gran Canaria. cperez@marinebiotechnology.org)

CFP outbreaks have occurred several years during the last decade in some (Lanzarote, Fuerteventura) of the Canary Islands. In all cases, the affected consumers had eaten "medregal" (*Seriola rivoliana*) caught either from sport or artisanal fishers, and served in local restaurants of these highly touristic area. The outbreaks had a strong socio-economic impact and affected local fish sales in an alarmed population with not much information on the problem. Following the last outbreak in Lanzarote (April 2012) local authorities prohibited the consumption of *Seriola* specimens larger than 15 kg and warned the population that all big fish have to be analysed by the Food Safety Department. To carry out these analyses, the fish needs to be frozen awaiting for the results of the analyses from samples sent to the capital of the archipelago (Gran Canaria), or even to the EU Reference Laboratory on Marine Biotoxins in Vigo.

Between May and November 2011, *Lyngbya majuscula*, a toxic benthic filamentous marine cyanobacterium that may cause dermatitis and other skin irritations, formed a bloom covering hundreds of square kilometres at a depth of 2-30 m off the east coast of Fuerteventura Island, Spain. This is the first record of this type of bloom in the Canary Islands. It was identified by BEA reported to the locally authorities.

2.2 Benthic HABs in Portugal

(Teresa Moita, IPMA, Lisbonne, Portugal tmoita@ipma.pt)

2.2.1 Mainland Portugal

As a consequence of the spreading of *Ostreopsis* blooms in the western Mediterranean Sea, a sampling programme was initiated in mainland Portuguese coastal waters aiming at the early detection of *Ostreopsis* and other benthic HAB species. The first surveys were carried out in 2007 (S coast) and 2008 (S and SW), and from all the samples analysed, specimens were only detected in Sines (SW) and identified as *Ostreopsis* cf. *siemensis* (Amorim *et a*l. 2010). Later benthic surveys identified the same species further north (David *et al*. 2012). Toxin profiles of the Atlantic strains were investigated and O. cf. *siemensis* seem to present a much lower risk to human health than *O*. cf. *ovata.* (Ciminello *et al.* 2013).

In September 2011, mucilaginous filaments were observed in seawater of D. Ana beach (Lagos coast, south Portugal). Genetic analyses of isolates revealed the bloom was due to the outbreak of *Ostreopsis* cf. *ovata* that reached $5.5x10^3$ cells L^{-1} in seawater samples, although concentrations remained lower (up to 320 cells L^{-1}) in adjacent areas (David *et al*., 2012). Local authorities closed several beaches for bathing for 5 days once informed about the bloom occurrence and model predictions for its transport. Only one case of respiratory and skin irritation was reported.

2.2.2 Azores and Madeira island's

Ostreopsis species were identified in the Azores archipelago (36-39ºN, 25-31ºW), at São Miguel island, during a summer survey carried out in 2008 (Silva *et al*., 2010). The averaged SST was above 21ºC around the island. The morphological analysis of the species suggested the presence of *O. heptagona, O.* cf. *siamensis* and *O.* cf. *ovata* that were present in seawater samples in small numbers (maximum 90 cells L^{-1}).

In Madeira island, *Ostreopsis* was detected for the first time in 2002 (Fraga, 2005), and the species identified was *Ostreopsis* cf. *ovata* (Penna *et al*., 2010). Further south, in Savage Islands, the genus was detected in seawater, in low concentrations (700 cells L^{-1}), only in summer 2008. A recent study on seaweeds around Madeira island revealed a maximum of 12,639 cells g^{-1} w.w. on *Corallina*/turf (Kaufmann and Bohm-Beck, 2013).

The above mentioned samples, collected at Savage Islands in 2008, were in fact sent to IPMA for HAB species identification after a severe incident of ciguatera-like intoxication affecting several fishers who consumed a big fish of *Seriola* sp. caught around the islands. At that time, despite the presence of *Ostreopsis*, no cells of *Gambierdiscus* were identified in seawater. Ever since, several CFP like episodes were reported almost every year and several ciguatoxins were detected from *Seriola* spp. caught in Savage Islands and Madeira (Vale, 2010; Boada, 2010). Since 2010, the regional authorities have banned the sale of *Seriola* spp. specimens greater than 10kg. Only in 2012, for the first time, Kaufmann and Böhm-Beck (2013) were able to identify *Gambierdiscus* sp. in seaweeds of Madeira. The identification of the species is under study.

2.3 Research and management of Ciguatera Fish Poisining in the US.

(Information provided by Pat Tester and D.M. Anderson)

There are two ongoing research projects in the US.

"*Species and Strain Differences in the Toxicity of Caribbean* Gambierdiscus *Species: Implications for Ciguatera Fish Poisoning in the Caribbean*" (pat.tester@noaa.gov). Ciguatera fish poisoning (CFP) is caused by the bioaccumulation of toxins, produced by tropical dinoflagellates in the genus *Gambierdiscus* when these toxins enter the marine food via herbivorous and then carnivorous fish. Globally, CFP causes more human illness than all other harmful algal bloom species combined. Despite this, relatively little is known about the variety of toxins produced by different *Gambierdiscus* species and the overall differences in toxicity among and between species. This is crucial information for understanding the variability of CFP incidences and how they may differ seasonally and spatially, even from the Atlantic to the Pacific. For example, ciguatoxins (CTXs) isolated from Atlantic and Pacific fish are structurally different. It has been proposed that these differences are due to the production of different toxin precursors by the resident *Gambierdiscus* species. Supporting evidence of this hypothesis comes from a recent study that confirmed distributional differences in *Gambierdiscus* species found in each basin. These differences may also account for why CFP symptoms tend to vary between the two regions. For example in the Pacific, *G. polynesiensis* is generally far more toxic on a per cell basis than other co-occurring species. If this is also true for the Caribbean, then it may be possible to assess CFP potential by monitoring these key species. We hypothesize that relatively few *Gambierdiscus* species are highly toxic and that they contribute disproportionately to the flux of toxins that enter the food chain. If this hypothesis is correct, species composition will affect the severity and frequency of CFP occurrences in an area. Given the importance of understanding the toxicity of individual *Gambierdiscus* species, the primary goals of this proposal are to:

- Establish the relative per cell toxicity of the five known Caribbean *Gambierdiscus* species and *Gambierdiscus* ribotype II using genetically characterized clones already established in the Tester (NOAA) Lab culture collection.
- Screen Caribbean *Gambierdiscus* species using the receptor binding bioassay to determine if *Gambierdiscus* ribotype 2 is more toxic than the other Caribbean species.
- Measure differences in hemolytic activity among Caribbean *Gambierdiscus* species as an indicator of MTX and CTX toxicity.
- Characterize and compare the toxins produced by Caribbean isolates of *G. caribaeus* from both the Caribbean and Pacific to determine if they produce different or similar toxin suites.
- Assess the extent to which CTX toxicity varies within a species and whether per cell toxicity varies systematically with latitude or geographic region.

Progress to date:

Detailed studies were undertaken using the hemolytic assay (Holland et al. 2013). Extensive fractionation studies were conducted to ensure the water soluble matitotoxin (MTX) and lipid soluble ciguatoxin fractions were being completely separated. The resulting protocol now provides a clean separation of the toxin types. This allows characterization of the variations in hemolytic activity (HA) from the CTX and MTX fractions extracted from six *Gambierdiscus* species found in the Caribbean. The results

from testing 56 isolates indicate that certain species had significantly greater HA than others. All species tested showed measurable HA with the exception of *G. carolinianus* isolates collected off the coast of North Carolina, USA. Experiments using specific inhibitors, hydrophilic and hydrophobic cell extracts, and heat treatments were all consistent with the observed hemolytic activity being due to maitotoxins (MTXs), the large polyether toxins produced by *Gambierdiscus* species. Geographically dispersed isolates of *G. caribaeus* (n = 26) and *G. carolinianus* (n = 15) were examined to determine regional differences in HA. *Gambierdiscus carolinianus* isolates from the western Caribbean and Gulf of Mexico had tenfold higher HA than isolates from the eastern Caribbean and 100-fold higher HA than isolates obtained from the continental shelf off North Carolina, USA. Similarly, *G. caribaeus* isolates from the western Caribbean and Gulf of Mexico had twofold higher HA than isolates from the Eastern Caribbean. Intra specific variation in HA of isolates from the same region was less than 5%. Depending on the species, HA consistently increased by ~7 to 40% from mid log growth phase to late log phase, and then declined in mid stationary phase to levels below those observed in log phase growth. Increasing growth temperatures from 20 to 31 °C for *G. caribaeus* from different regions showed only a slight increase in HA between 20 and 27 °C. HA in the *G. carolinianus* isolates from different regions grown over the same 20 to 31 °C temperature range remained constant. These data suggest that growth temperature is not a significant factor in modulating the intra and inter specific differences in HA. The HA of various isolates measured repeatedly over a 2 year period remained constant, indicating the compounds responsible for haemolysis were constitutively produced and under strong genetic control. Depending on species, > 65 to 95% of the total HA was associated with the cell membranes. The variation in HA for a limited number of *Gambierdiscus* isolates tested from outside the Caribbean was similar to that found within the Caribbean. This suggests that variations in HA among *Gambierdiscus* species is similar worldwide.

Another ongoing study in the US called *CiguaHAB* (danderson@whoi.edu) has the following objectives: 1) To characterize *Gambierdiscus* population diversity and connectivity on regional and local scales; 2) to determine effects of environmental factors on the growth and toxicity of representative strains of *Gambierdiscus*; 3) to investigate *Gambierdiscus* population dynamics and the environmental conditions that contribute to blooms in several locations for the study region; 4) to investigate the fate of ciguatoxins, their precursors, and metabolites in the coral reef foodweb; and 5) to model the population dynamics and toxin production of *Gambierdiscus* under different environmental forcing, including those associated with natural and human-induced perturbations.

At the federal level, the US FDA has the most extensive ciguatera-related activities in the US, though there are some research and monitoring efforts in other agencies such as the National Oceanic and Atmospheric Administration (NOAA) and the Centres for Disease Control (CDC). FDA activities (including collaborative research) at the Gulf Coast Seafood Laboratory, Dauphin Island, Alabama, include the following:

Outbreak Response and Training

Goal: Assist and respond to outbreaks of CFP, evaluate incidence, and identify new regions of concern

1) Assist international, national (e.g. CDC), state, and regional health departments in outbreak response for ciguatera fish poisoning. Includes consultation and guidance on sampling and information gathering, analysis of fishmeal remnants for ciguatoxins, and fish species identification as need-

ed. These efforts involve several divisions within FDA including the Division of Seafood Science and Technology (GCSL), Division of Seafood Safety (policy and guidance), and trace back with FDA district offices and the Coordinated Outbreak Response and Evaluation (CORE) Network.

2) Fish import surveillance testing as needed following reports of outbreaks in new/emerging areas of concern

3) Provide agency, interagency, state, and international training on chemical methods for ciguatoxins

Methods and Standards**:**

Goal: Optimize recovery, streamline analysis and validate methods for CFP

1) Validate screening and confirmatory methods for the detection and quantification of ciguatoxins in fish, shellfish, crustaceans, and clinical samples.

2) Oaubain- veratridine dependent neuroblastoma cytotoxicity assay (sodium channel specific)

3) Liquid chromatography-tandem mass spectrometry methods

4) Develop and optimize chemical extraction and solid phase clean-up methods for ciguatoxins in seafood and clinical matrices

5) Isolate, purify, and produce ciguatoxin reference materials to support FDA regulatory and research analysis

6) Evaluate methods that are commercially available, develop new methods as technology and data becomes available

Prevalence of CFP and Distribution of Ciguatoxins

Goal: Gain better understanding on the prevalence and distribution of ciguatoxins in fish on a regional scale to prevent illness and inform relevant stakeholders

1) Prevalence of ciguatoxins in commercially relevant species

1.1) Virgin Islands and Florida predatory reef fish

1.2) Evaluate the potential human health risk associated with harvest and consumption of invasive species (e.g. lionfish)

1.3) Flower Garden Banks National Marine Sanctuary with NOAA NMS (Emma Hickerson)

2) Perform fish market surveys to evaluate and identify risk and background exposure to ciguatoxins in endemic regions

Bioaccumulation and Trophic Transfer

Goal: Characterize the uptake, bioaccumulation, and elimination of ciguatoxin precursors, toxins, and metabolites within and between species

1) Ciguatoxin exposure experiments in model fish and reef species

2) Determine the tissue distribution of ciguatoxins in fish to determine best sampling practices

2.1) Caribbean

2.2) Pacific (collaboration)

3) LC-MS/MS profiling and metabolomics of benthic dinoflagellates and fish from multiple trophic levels

Biomarkers of exposure

Goal: Identify characteristic biomarkers of ciguatoxin exposure in fish and clinical samples to develop new methods, field, and diagnostic tests

1) Transcriptomics and proteomics of clinical samples from CFP exposed individuals from the US Virgin Islands

2) Proteomics and transcriptomics analysis of fish following incurred exposure to ciguatoxins and assessment of toxic and non-toxic fish from a single species.

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3.6 ToR g) Finalize plans for Workshop on Automated Harmful Algal Bloom in situ Observation Systems

During the prior WGHABD meeting in Oban, the idea of holding a workshop on in situ, autonomous sensors for HAB cells and toxins was discussed. The original goal was to acquaint Working Group members with the new technologies, as well as with the realities of purchasing, maintaining, and operating them in HAB research and monitoring programs. The ideal place for such a meeting would be in Woods Hole, since the Anderson lab owns two of the major instruments that were to be evaluated – the Environmental Sample Processor (ESP) and the Imaging Flow Cytobot (IFCB).

The ESP is a submersible, robotic instrument that collects discrete water samples, concentrates micro-organisms or particles, extracts target molecules, and automates application of molecular probes to identify micro-organisms and gene products. Real-time detection chemistries rely on DNA probe and protein (antibody) arrays to detect target molecules such as the HAB toxin domoic acid. Potential applications of ESP are many, as corroborated in the short video found at http://www.mbari.org/ESP/espdeepmovie.htm. In comparison to the IFCB's output, ESP data products are much less information rich but far more definitive, giving species-specific abundance estimates of tens of target molecules for up to 44 time points during a single deployment. The limited number of time points is a constraint imposed by the system's array magazine.

The IFCB is an imaging flow cytometer that essentially acts as an automated, underwater microscope. It is able to analyse one 5-mL seawater sample every 20 minutes, taking up to 12 images s^{-1} of phytoplankton cells ranging in length from 10 to 100 μm.