Intergovernmental Oceanographic Commission
Training Course Reports

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IOC-UNEP-WHO-FAO
Training Course on Toxin Chemistry and Toxicology related to Harmful Algal Blooms

Organized with the support of the Government of Italy

University of Trieste
Trieste, Italy
3-12 September 1995

UNESCO

IOC Training Course Reports

No.	Title	Language versions
1.	IOC Indian Ocean Region Training Course in Petroleum Monitoring Perth, 18 February-1 March 1980	English
2.	IOC Regional Training Course for Marine Science, Technicians Cape Ferguson, Queensland, 1-28 June 1980	English
3.	ROPME-IOC-UNEP Training Workshop on Oceanographic Sampling Analysis, Data handling and Care of Equipment, Doha, Qatar, 3-15 December 1983	English
4.	Stage COI d'initiation à la gestion et au traitement de l'information scientifique et technique pour l'océanologie, Brest, France, 28 novembre - 9 décembre 1983	French
5.	Curso mixto COI-OMM de formación sobre el Sistema Global Integrado de Servicios Oceánicos (SGISO), Buenos Aires, Argentina, 15-26 de octubre de 1984	Spanish
6.	UNESCO-IOC-NBO Training Course on Tidal Observations and Data Processing Tianjin, China, 27 August - 22 September 1984	English
7.	Stage COI sur la connaissance et la gestion de la zone côtière et du proche plateau continental Talence, France, 18 septembre - 4 octobre 1984	French
8.	IOC Regional Training Course on Marine Living Resources in the Western Indian Ocean Mombasa, Kenya, 27 August - 22 September 1984	English
9.	IOC-UNESCO Summer School on Oceanographic Data, Collection and Management Erdemli, Icel, Turkey, 21 September - 3 October 1987	English
10.	IOC-UNESCO Regional Training Workshop on Ocean Engineering and its Interface with Ocean Sciences in the Indian Ocean Region, Madras, India, 17 March - 5 April 1986	English
11.	IOC-UNESCO Training Course on the Use of Microcomputers for Oceanographic Data Management Bangkok, Thailand, 165 January - 3 February 1989	English
12.	IOC Advanced Training Course on Continental Shelf Structures Sediments and Mineral Resources Quezon City, Philippines, 2-13 October 1989	English
13.	IOC/IODE Training Course on GF3 Data Formatting System Obninsk, USSR, 14-24 May 1990	English
14.	IOC Training Course on Microcomputers and Management of Marine Data in Oceanographic Data Centres of Spanish-speaking Countries, Bogotá, Colombia, 21-30 October 1991	English Spanish
15.	IOC Advanced Training Course on Nearshore Sedimentation and the Evolution of Coastal Environments, Kuala Lumpur, Malaysia, 17-29 February 1992	English
16.	First IOC Training Course on the Applications of Satellite Remote Sensing to Marine Studies Caracas, Venezuela, 24-28 September 1990	English
17.	IOC-KMFRI-RECOSCIX (WIO) Regional Training Course on Microcomputer-based Marine Library Information Management, Mombasa, Kenya, 10-21 August 1992	English
18.	ROPME-IOC Regional Training Course on Management of Marine Data and Information on Microcomputers for the ROPME Region, Kuwait, 18-28 October 1992	English
19.	IOC-SOA Training Workshop on Environmental Effects on Benthic Communities Xiamen, China, 19-23 October 1992	English
20.	IOC Training Course for the Global Sea Level Observing System (GLOSS) directed to the African and South American Portuguese and Spanish-Speaking Countries São Paulo, Brazil, 1-19 February 1993	English
21.	IOC-SSTC-SOA Training Course on Marine Information Management and ASFA Tianjin, China, 19-30 October 1992	English
22.	First IOC/IOCARIBE-UNEP Training Course on Monitoring and Control of Shoreline Changes in the Caribbean Region, Port-of-Spain, Trinidad and Tobago, 21-30 July 1993	English Spanish
23.	IOC/WESTPAC Training Course on Numerical Modelling of the Coastal Ocean Circulation Matsuyama, Japan, 27 September - 1 October 1993	English
24.	IOC-JODC Training Course on Oceanographic Data Management Tokyo, Japan, 28 September - 9 October 1992	English
25.	IOC-JODC Training Course on Oceanographic Data Management Tokyo, Japan, 27 September - 8 October 1993	English
26.	IOC Training Course on Ocean Flux Monitoring in the Indian Ocean. Organized with the support of the Government of Germany, Mombasa, Kenya, 15-27 November 1993	English
27.	IOC-UNEP-SPREP Training Course on Coral Reef Monitoring and Assessment Rarotonga, Cook Islands, 23 February - 13 March 1994	English
28.	IOC-JODC Training Course on Oceanographic Data Management Tokyo, Japan, 28 September - 9 October 1992	English
29.	IOC-UNEP-WHO-FAO Training Course on Qualitative and Quantitative Determination of Algal Toxins Jena, Germany, 18-28 October 1994	English
30.	IOC Training Course on Oceanographic Data Management for the Black Sea Countries Obninsk, Russian Federation, 1-12 August 1994	English
31.	COI-CEADO Curso Regional de Capacitación en Gestión de Datos e Información Oceanográficos Buenos Aires, Argentina, 17-28 de octubre de 1994	Spanish
32.	IOC-UNEP-FAO Training Course on Nutrient Analysis and Water Quality Monitoring Zanzibar, Tanzania, 21-26 November 1994	English
33.	IOC-IOMAC Advanced Training Course on Marine Geology and Geophysics off Pakistan. Organized with the support of the Government of Germany National Institute of Oceanography. Karachi, Pakistan, 12-26 November 1994	English
34.	Training Course on Management of Marine Data and Information for the Mediterranean Region Valletta, Malta, 10-21 April 1995	English
35.	IOC-UNEP-WHO-FAO Training Course on Toxin Chemistry and Toxicology related to Harmful Algal Blooms	English

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ABSTRACT

The *IOC-UNEP-WHO-FA O-Italy Training Course on Toxin Chemistry and Toxicology related to Harmfud Algal Blooms* was held at the University of Trieste, Italy, from 3 to 12 September 1995. The Course was jointly sponsored by the University of Trieste (Italy), the Intergovernmental Oceanographic Commission (IOC) of UNESCO, the United Nations Environmental Programme (UNEP), the World Health Organization (WHO), the Food and Agricultural Organization of the United Nations (FAO), as well as by other Institutions and Companies as reported in Chapter 6.

The Course was organized by Dr. Aurelia Tubaro (University of Trieste, Italy) and by Prof. Roberto Della Loggia (University of Trieste, Italy) in co-operation with Dr. Helle Ravn (IOC, Paris, France) and Prof. Giorgio Honsell (University of Udine, Italy), and hosted by the Department of Biomedical Sciences, University of Trieste, Italy, Dr. A.D. Cerebella (Institute for Marine Biosciences, Halifax, Canada), Dr. M.L. Fernandez (European Communities Reference Laboratory on Marine Biotoxins, Vigo, Spain), Dr. A. Martinez Fernandez (European Communities Reference Laboratory on Marine Biotoxins, Vigo, Spain), Prof. J.S. Ramsdell (Southeast Fisheries Science Center, South Carolina, USA), Dr. Helle Ravn (IOC, Paris, France) and Prof. T. Yasumoto (Tohoku University, Sendai, Japan) were invited to give lectures. The participants were 13, coming from 12 different countries (Annex H).

The Course was held in order to improve the theoretical and practical knowledge of the participants on detection methods of toxins produced by microalgae and responsible for the most widespread human intoxication due to the consumption of shellfish (DSP, PSP and ASP), In particular, the Course was focused on methods that do not require complex and expensive equipment, not always available in the countries of the participants.

During the Course a basic knowledge on the taxonomy and distribution of the microalgae responsible for these kinds of intoxication was given. Furthermore, the identification of toxic microalgae, both in seawater and in the stomach content of mussels, was demonstrated.

Seven different methods for the detection of toxins were carried out by each participant or demonstrated to them by the lecturers. In particular, DSP contaminated samples were analyzed by the participants, using three different methods: a mouse bioassay, an ELISA assay and a protein phosphatase inhibition assay. The HPLC method for DSP toxins was also demonstrated.

PSP contaminated mussels were analyzed using the AOAC mouse bioassay. The same samples. as well as other contaminated specimens (i. e. microalgae and urine of PSP patients) were analyzed also by a receptor assay. An ELISA assay for PSP was also demonstrated.

The results obtained by each participant were compared and discussed during two round tables and a satisfactory correlation was found among the data obtained with the different methods. The practical aspects of all the applied methods were emphasized to enable the participants to perform the analyses in their laboratories.

All the participants gave a report about their national situation on Harmful Algal Blooms. In particular, during a round table the different legislations adopted in the countries of the participants were discussed.

1. BACKGROUND

During the last decades, the occurrence of toxic algal blooms has increased all over the world. The algal blooms are not only an environmental, but also a food and health problem. Toxic phytoplankton imposes serious problems on fisheries and aquiculture. The toxins produced by the algae accumulate in shellfish or fish through the marine food chains, leading to severe human poisoning. Furthermore, they can cause massive kills of fish or shellfish.

Monitoring programmes have been developed to avoid human poisoning. In many countries, the monitoring programmes include controls of shellfish toxicity and of toxic algae presence in seawater as major parameters for the management of aquiculture. The collection of shellfish is prohibited if toxic algae are above certain concentration limits and/or if shellfish contamination works above the tolerance limits fixed by national regulation. However, there is no simple correlation between the concentration of algae in seawater and the concentration of algal toxins in shellfish. Indeed, shellfish may contain algal toxins above the tolerance limit for human consumption, even if low concentrations of toxic algae are detected in seawater.

The assessment of phycotoxins often represents a challenge due to complexity of the matrix represented by shellfish. It is therefore very important with recurrent training of chemists and biologists in the analysis and handling of toxins from shellfish and phytoplankton. In many countries training on analytical methods for the detection of phycotoxins are needed to ensure reliable toxicological and chemical analyses.

Needs for training activities in the field of toxin chemistry and toxicology were expressed at the BTMC-IOC-POLAMAR International Workshop on Training Requirements in the field of Eutrophication in Semi-Enclosed Seas and Harmful Algal Blooms, Bremerhaven, Germany, 29 September-3 October, 1992. At the Second Session of the IOC-FAO Intergovernmental Panel on Harmful Algal Bloom, Paris, 14-16 October 1993, the needs for global training activities were confirmed and identified and a Training Programme on Harmful Algae was accordingly adopted.

The first Course in this field, the IOC-WHO-FAO Training Course on Qualitative and Quantitative Determination of Algal Toxins, was organized at Friedrich-Schiller University of Jena, Germany from 18 to 28 October 1994. The Course focused on the HPLC determination of algal toxins. However, since the HPLC equipment is expensive and requires trained operators, many developing countries can not carry out this kind of analysis. Therefore, there is a strong need to organize training courses on the official or accepted test methods, such as the mouse bioassay, and on alternative methods less sophisticated and less expensive than HPLC. The needs to organize valuable and reliable monitoring programmes and research projects in the field of algal toxins were identified during the above mentioned Course in Germany.

2. GOALS OF THE COURSE

The Course was focused on the improvement of the experience of the participants in the field of toxicology and toxin chemistry related to algal toxins both from a theoretical and a practical point of view.

One of the aims of the Course was to give an overview on the main intoxications (PSP, DSP and ASP) due to the ingestion of contaminated shellfish considering the causative agents, the chemistry of the toxins involved, eir toxicological effects and the clinical symptoms.

Particular attention was paid to the actual situation of analytical methods developed for toxin analysis as well as to the toxins responsible for the poisonings observed in the countries of the participants.

Another important goal was for the participants to learn to apply the basic methods of toxin analysis together with new, rapid and not expensive methods for the most widespread intoxications (DSP and PSP).

A further aim of the Course was to establish research links among the participants, the lecturers and the host Institution to amplify the international co-operation.

3. CONTENT OF THE COURSE

The Course was related to the problems of the harmful algal blooms in the countries of the participants with particular reference to the methods used to evaluate the toxicological risk.

The Course included a theoretical and a practical part. The theoretical part gave the participants an overview of the main intoxications (DSP, PSP, ASP, NSP, CFP) considering the causative agents and the toxins responsible from a chemical, biological and toxicological point of view (see Annex V),

The practical part had the aim to enable the participants to use different methodologies. The assessment of toxicological risk was performed using the following methods (see Annex VI):

(i) for the detection of DSP contamination in mussel samples, the Mouse Bioassay, an immunoenzymatic assay (ELISA) and a protein phosphatase inhibition assay were performed; a demonstration of the HPLC analysis was also given;

(ii) for the detection of PSP toxicity, the AOAC Mouse Bioassay was performed on mussel samples and a receptor assay was performed on shellfish, algae, and human urine samples: an ELISA assay was also demonstrated.

A practical demonstration of the identification of toxic microalgae, both in seawater and in the stomach content of mussels, was also given.

4. NATIONAL REPORTS

The participants presented National Reports with regard" to the poisonings observed in their country, the microalgae involved, the toxins related to the events, the shellfish or finfish involved, the rules or laws stated by their Governments for the control of shellfish and/or finfish contamination (see Annex 111),

5. EVALUATION AND CONCLUSION

The scientific background and the practical experience of the participants on toxic chemistry and toxicology related to HAB was variable. The majority of them came from countries where monitoring networks and controls for phycotoxin contamination of seafood are not effective or are only beginning. Only some of them were already involved in phycotoxin controls. The global spreading of HAB, related toxic events and the development of aquiculture prompts many countries to set up control systems of seafood algal contamination. With this in mind the course gave the theoretical and practical bases for the detection of phycotoxins in shellfish containing the most widespread human intoxications (PSP, DSP and ASP), The course was focused on biological tests (mouse bioassay) which are the official tests for the determination of toxicity in most countries and on new high sensitivity tests that do not require expensive equipment and can be easily applied.

All the participants performed successfully different tests and received information on all the main aspects necessary to set up the tests in their countries. The evaluation of the results allowed to focus the main problems arising in the application of different tests and in the interpretation of results to the toxicological risk.

The discussion among participants and teachers was productive and identified specific problems encountered by the participants and often provided solutions. An important result of the course was given by the contacts established which, together with the information and the practical experience achieved, could be the basis for networks of control in new countries and co-operative research programmes.

6. ACKNOWLEDGEMENTS

The Training Course was hosted and co-sponsored by:

University of Trieste, Italy
Department of Biomedical Sciences
University of Trieste, Italy.

The Training Course was co-sponsored by:

Intergovernmental Oceanographic Commission (IOC of UNESCO)
United Nations Environment Programme (UNEP)
Food and Agricultural Organization (FAO)
World Health Organization (WHO).

Scientific, technical equipment, reference materials and financial support were also given by:

Department of Health and Human Services, Food and Drug Administration - Washington DC, USA.
Institute for Marine Biosciences, National Research Council of Canada - Halifax, Canada
Southeast Fisheries Science Center, National Marine Fisheries Service - Charleston, SC, USA
European Community Reference Laboratory of Vigo - Spain
Analitica s.n.c. - Trieste, Italy
Gilson Italia s.r.l. - Milano, Italy
Gilson Medical Electronics France S.A. - Villiers-Le-Bel, France
Leica S.p.A. - Milano, Italy
Panapharm Laboratories Co. Ltd. - Kumamoto, Japan
Perkin-Elmer Italia S.p.A. - Padova, Italy.

Facilities and further economical support were supplied by:

Azienda di Promozione Turistica (APT) di Trieste - Italy
Comune di Trieste - Trieste, Italy
Camera di Commercio, Industria, Artigianato ed Agricoltura (C. C. I. A. A.) di Trieste - Italy
Cassa di Risparmio di Trieste Fondazione - Trieste, Italy
Aboca s.r.l. - San Sepolcro, Arezzo, Italy
Consorzio Volontario Interregional Produttori Organismi Marini Alto Adriatico - Trieste, Italy
Illycaffè - Trieste, Italy
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ANNEX I

DETAILED PROGRAMME

Sunday September, 1995

Arrival of participants

Monday September 4,1995

9:00 -- 9:30 a.m. **Welcome to the participants - Opening Ceremony:**

Dr. R. Illy, Major of Trieste, gave the welcome of the town of Trieste to the participants.

Prof P. Linda Dean of the Faculty of Pharmacy of the University of Trieste, gave the welcome of the University of Trieste to the participants and underlined the importance of the Course.

Dr. G. D'Orlandi, Councillor of Environment of Friuli Venezia Giulia Region, gave the welcome of the Friuli Venezia Giulia Government to the participants.

Dr. H. Ravn, IOC of UNESCO, presented the activities of the Intergovernmental Oceanographic Commission of UNESCO.

Dr. Tubaro gave technical and practical information on the Course.

9:30 - 10:00 a.m. Coffee break

10:00 -11:00 a.m. **Lecture**

Dr. A. Tubaro (Italy): Different kinds of poisonings related to toxic algae.

(With particular attention to clinical symptoms, therapy and diagnosis of DSP, PSP and ASP).

11:00 - 12:00 a.m. **Lecture**

Prof. G. Honsell (Italy): Harmful Algae.

(General introduction on the biology, taxonomy, ecology and distribution of potentially toxic microalgae).

12:00 - 12:30 a.m. **Lecture**

Prof. G. Honsell (Italy): Causative agents of Diarrhetic Shellfish Poisoning.

(With special reference to the genus Dinophysis).

2:00 - 3:30 p.m. **Lecture**

Prof. T. Yasumoto (Japan): DSP and NSP toxins.

(Toxins responsible for DSP and NSP, their chemical conversion in shellfish and degradation processes).

3:30 - 4:00 p.m. Coffee break.

4:00 - 6:30 p.m. Round Table

National Reports,

(The participants presented National Reports with regard to the poisoning detected in their country, the microalgae involved, the toxins related to the events, the shellfish or finfish involved, the rules or laws stated by their Governments for the control of shellfish and/or finfish contamination).

Tuesday September 5,1995

9:00 - 10:30 a.m. Lecture

Prof. T. Yasumoto (Japan): Analytical methods for determination of DSP and other toxins in shellfish, algae and seawater,

(A survey on the main methods (HPLC, LC/MS, ELISA) to DSP and other toxins in shellfish and algae. Emphasis was given on new sensitive methods to detect the toxins also in seawater).

10:30 -11:00 am. Coffee break.

11:00 - 12:00 am. Lecture

Prof. R Della Loggia (Italy): Experimental determination of DSP toxins using the mouse bioassay.

(The correct application of Mouse Bioassay was presented considering its advantages and disadvantages).

2:00 - 7:00 p.m. Practice.

Dr. S. Sosa, Dr. L. Cerni. Mouse bioassay for DSP toxins: preparation of the samples.

(Participants were divided in groups A, B, C, D, E, F. Each participants prepared one extract of DSP contaminated mussels).

Wednesday September 6,1995

Groups A, B, C.

8:30 a.m.- 6:30 p.m. Practice.

Prof. R Della Loggia and Dr. S. Sosa: Practical determination of DSP toxins by mouse bioassay and evaluation of the results,

(Each participant performed the mouse bioassay according to the Italian law).

Demonstration.

Prof. G. Honsell, Dr. P. Nichetto and Dr. L. Sidari: Identification of toxic algae.

(A demonstration of the identification of Dinophysis and Alexandrium species in seawater was done).

Groups D, E, F

9:00 a.m. - 1:00 p.m. Practice.

Dr. A. Tubaro and Dr. L. Cerni: Determination of DSP toxins using ELISA.

(A description of the assay with its advantages and disadvantages was presented. Each participant prepared the DSP contaminated mussel extracts that were tested by the ELISA).

2:30 - 7:30 p.m. Practice.

Dr. C. Florio and Dr. E. Luxich: Determination of DSP toxins by a protein phosphatase 2A inhibition assay.

(A description of the assay, based on the mechanism of action of OA, with advantages and disadvantages, was provided. Each participant prepared the mussel extracts that were tested by PP2A inhibition assay).

Thursday September 7,1995

Groups D, E, F.

8:30 a.m. - 6:30 p.m. Practice.

Prof. R Della Loggia and Dr. S. Sosa Practical determination of DSP toxins by mouse bioassay and evaluation of the results.

(Each participant performed the mouse bioassay according to the Italian law).

Demonstration.

Prof. G. Honsell, Dr. P. Nichetto and Dr. L. Sidari: Identification of toxic algae.

(A demonstration of the identification of Dinophysis and Alexandrium species in seawater was done).

Groups A, B, C.

9:00 sm. - 1:00 p.m. Practice.

Dr. A. Tubaro and Dr. L. Cerni: Determination of DSP toxins using ELISA.

(A description of the assay with its advantages and disadvantages was presented Each participant prepared the DSP contaminated mussel extracts that were tested by the ELISA).

2:30 - 7:30 p.m. Practice.

Dr. C. Florio and Dr. E. Luxich: Determination of DSP toxins by a protein phosphatase 2A inhibition assay.

(A description of the assay, based on the mechanism of action of OA, with advantages and disadvantages, was provided. Each participant prepared the mussel extracts that were tested by PP2A inhibition assay).

Friday September 8, 1995

9:00 - 10:30 a.m.	Round Table.
	Prof. R. Della Loggia: Practical evaluation of the potential toxicological risk of DSP.
	(Guided discussion and comparison of the results obtained with the three different methods by each participant).
10:30 - 11:00 a.m.	Lecture.
	Prof. G. Honsell (Italy): Causative agents of Paralytic Shellfish Poisoning.
	(With particular reference to Alexandrium, Gymnodinium and Pyrodinium species).
11:00 -11:30 a.m.	Coffee break.
11:30 a.m. - 1:00 p.m.	Lecture.
	Dr. H. Ravn (France): Toxicological and chemical aspects of Paralytic Shellfish Poisoning
	(An overview of the PSP symptoms, responsible toxins, analytical methods and pharmacological treatment).
2:30 - 6:30 p.m.	Practice.
	Dr. A. Martinez: Preparation of the samples for PSP toxins determination by the AOAC mouse bioassay.
	(Each participant prepared the PSP contaminated mussel samples, according to the AOAC procedure).

Saturday September 9,1995

9:00 - 10:30 a.m.	Lecture.
	Prof. J. S. Ramsdell (USA): The use of receptor assays for the detection of phycotoxins.
	(The theoretical basis of the method was explained together with the main advantages and disadvantages).
10:30 -11:00 a.m.	Coffee break.
11:00 - 12:30 a.m.	Demonstration.
	Prof. J. S. Ramsdell: Receptor assay for PSP:first part.
	(A practical application of the assay to PSP contaminated mussels, algae and urine samples).
2:30 - 3:30 p.m.	Demonstration.
	Prof. J. S. Ramsdell: Receptor assay for PSP: second part.
3:30 - 4:30 p.m.	Lecture.
	Dr. A. D. Cerebella (Canada): Determination of PSP toxins using an ELISA assay.
	(The basis of the method was presented and a demonstration was given).

4:30 - 5:30 p.m.	Round Table.
	Problems of standardisation of toxin analysis: current legislation in various countries.
	(A survey of the regulation in the countries represented at the Round Table was given and the features of an optimal legislation were discussed).
6:00 - 8.00 p.m.	Guided tour to Rosenquist art exhibition (Museo Revoltella).
09:00 p.m.	Dinner to the 'Arco di Riccardo " restaurant.

Sunday September 10,1995

9:00 a.m. - 3:15 p.m.	Excursion
	Boat visit to mussel-farms of the Gulf of Trieste.
	Lunch at Villaggio del Pescatore.
4:30 - 5:15 p.m.	Lecture.
	Dr C. Casadei (Italy): Introduction of HPLC analyses for DSP toxins.
5:15 - 7:30 p.m.	Demonstration.
	Dr C. Casadei: HPLC analysis of DSP toxins.
	(HPLC outputs for OA standard solutions and derivatized mussel samples were obtained)

Monday September 11,1995

8:30 - 9:30 a.m.	Lecture
	Dr. A. Martinez (Spain): The AOAC mouse bioassay for PSP.
	(The basis of the analytical procedure and the factors that can influence the results were presented).
9:30 a.m. - 1:00 p.m.	Practice.
	Dr. A. Martinez and Dr. S. Sosa: Determination of PSP toxins by AOAC mouse bioassay
	(Mice standardisation and PSP toxin determination in unknown mussel samples)
2:30 - 3:30 p.m.	Round Table
	Dr. A. Martinez and Prof. J.S. Ramsdell: Evaluation of the obtained PSP data.
	(The results obtained using the mouse bioassay and the receptor assay were discussed).
3:30 - 4:00 p.m.	Lecture
	Prof. G. Honsell (Italy): Algal species involved in Amnesic Shellfish Poisoning

	(The causative agents of ASP were presented, with reference to Pseudo-nitzschia species).
4:00 - 5:30 p.m.	Lecture
	Dr. M. Fernandez (Spain): Analytical methods for determination of ASP toxins.
	(With special attention to the HPLC method for the domoic acid determination).
5:30 - 6:00 p.m.	Coffee break.
6:00 - 6:30 p.m.	Lecture.
	Dr. L. Sidari (Italy): Sampling strategic for algae and mussels monitoring.
	(With particular emphasis on DSP contamination routine control)
6:30 - 7:00 p.m.	Closing ceremony
8:30 -11.00 p.m.	Course dinner at “Al Britannia’’ restaurant.

Tuesday, September 12,1995

Departure of participants.

ANNEX II

LIST OF PARTICIPANTS

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ANNEX III

NATIONAL REPORTS

ALBANIA

This presentation is rather difficult, because in Albania data on food poisoning in general and particularly from marine products such as shellfish and finfish are not available. Until now in Albania studies on the determination of toxic algae and their toxins in shellfish and /or in finfish are not performed. Hydrobiology practically is in the embrional stage. There is only a study where it is reported the presence of different dinoflagellates like Gymnodinium sp., Prorocentrum, Dinophysis, etc. which produce toxins that are accumulated in shellfish or finfish.

On the other hand, some work has been done, by the Institute of Public Health (IPH), on the evaluation of the microbiological quality of the waters as well as on the bacterial contamination of shellfish, that were cultivated and exported until 1990. Other analyses concerning toxins or chemical contamination were not carried out. For shellfish cultivated and exported until 1990, only the index of bacterial contamination was measured by the IPH, while the toxic and chemical contamination was not evaluated. During 1994-1995 a Swiss project for the rehabilitation of the Department of the Protection of Environment and Health will provide the IPH with contemporary equipment for the analysis of metals in all kind of products.

Meanwhile the Institute of Biological Research and the Faculty of Medicine (University of Tirana) are supplied with contemporary equipment like HPLC and spectrophotometers of high quality, but in spite of that, the efforts to contribute on the problems of the study for the protection of the environment, have not yet been realized because of the lack of the experience and scientific communications with developed countries.

Certainly, this course will open a new window that did not exist in Albania, not only with scientific interest but also with a practical one as well.

AUSTRIA

About ten to twenty years ago, eating shellfish was not very popular in Austria. Nowadays there is a certain market on these products because of the increasing tourism of Austrians to foreign countries. No cases of poisoning with marine toxins caused by shellfish are known in Austria.

PSP: Since 1977 PSP had been detected in canned shellfish only in three periods. In November 1977 Austria got a warning from Germany that PSP toxins had been detected in canned shellfish. In France as well as in Germany some severe poisonings occurred. The Federal Institute of Food Investigation and Research began to detect the paralytic shellfish poison at that time. PSP was detected in 1977-1978 and 1985-1986. In 1977-1978 some of those samples had values of more than 1000 µg of saxitoxin equivalents/l00 g in the whole content of the can. The PSP toxins were detected by analytical methods.

The reasons for using the fluorometric method at first and since 1992 the HPLC-method instead of the mouse bioassay for detecting the PSP are:

(i) Animal tests in Austria are restricted by the law for the protection of animals and an exception is required. It is very difficult to perform such tests for food because it is considered as a kind of luxury;

(ii) After the first positive tests the fluorometric method was compared with the mouse bioassay in a medical institute and the correlation was satisfactory.

In 1987 the mouse bioassay was used to verify the highest values found by the fluorometric method.

All the positive samples were canned blue mussels (Mytilus edulis). In 1993 most of the analysed shellfish came from New Zealand and belonged to the species Perna canaliculus.

DSP: DSP analysis had been done only on few samples per year with the HPLC-method developed by Luckas and co-workers. Until now, no positive samples were found.

ASP and NSP were not analysed until now.

Sampling: Sampling is done by civil servants who have special training in taking food samples. These are taken as spot checks. After receiving warnings or values above limit were found, special programs for collecting the samples were started. Since this laboratory is a part of the Federal Institute of Food Investigation and Research, all of the monitoring programs are official.

Since Austria is a member of the European Community, there is a new necessity of testing all the imported food from outside the EC. There are special veterinary programmes at the borders to investigate the imports of food from countries outside the EC. The inspectors (veterinarians) have to order the analyses according to a form given out by the Austrian Ministry of Health and Consumer Protection in qualified official investigation institutes.

CROATIA

In the Croatian Coastal waters, the Harmful Algal Bloom problem is present only in certain localities exposed to a higher level of anthropogenic eutrophication (Northern Adriatic, Sibenik Bay, and Kastela Bay). In the course of the last twenty years, monospecific blooms and red-tides have occurred in these localities. These blooms have been followed by a temporary local kill of sea fauna (hypoxia). Blooms of toxic and suspect toxic organisms began developing in the late 1980's and early 1990's, but those blooms were limited to highly eutrophicated areas.

In the coastal waters of the northern Adriatic, various species of the genus Dinophysis, especially Dinophysis fortii, have caused relevant problems. In the Sibenik Bay area, a very strong red-tide of Prorocentrum minimum develops every summer, while the highest number of toxic species is recorded in the Kastela Bay area. In this area, Dinophysis sacculus develops frequently, and Dinophysis fortii is sometimes found. The usual bloom of the non toxic species Gonyaulax polyedra has been followed by an Alexandrium minutum bloom in the past few years. In July, 1995, there was a development of a multi-species red-tide consisting of Gonyaulax polyedra ($> 1x10^7$ cells/1), Alexandrium minutum ($> 8x10^5$ cells/1), and Dinophysis sacculus ($8x10^4$ cells/1).

Since the end of 1993, the Institute of Oceanography and Fisheries has been doing shellfish analyses (DSP and PSP), which were performed by foreign laboratories earlier, and were mainly done on shellfish from shellfish farms. Toxicity has been recorded only in shellfish taken from the Kastela Bay. The toxicity never exceeded those levels permissible by law for PSP and DSP (the analysis of shellfish from July 1995 have not yet been done).

The monitoring of eutrophication (biological and chemical parameters) has been in effect for over twenty years in both the northern Adriatic (Center for Marine Research - Rovinj), and in the middle and southern Adriatic (Institute of Oceanography and Fisheries - Split). We started monitoring toxic phytoplankton species in 1989 in the form of the following national monitoring programs: Causes, dynamics and consequences of exceptional phytoplankton blooms and also in UNEP sponsored programs: Toxic phytoplakton species in the eastern Adriatic coastal waters (UNEP), and Investigation of growth and distribution of potentially toxic organisms in some shellfish forms (Middle Adriatic) (UNEP).

In June, 1994, the introduction of new laws for shellfish toxicity monitoring was a positive step taken by the Croatian government. Unfortunately, the health department is only legally required to perform tests on shellfish for import or export; our Institute monitors shellfish farms and does tests for scientific purposes only.

GEORGIA

In Georgia, which is one of the richest countries in the world for its water resources, the first harmful algal bloom was recorded in Paliastomi Lake in 1964. This Lake is located in the western part of the country, and it is a Black Sea coastal lagoon. Algal blooms have taken place periodically in this eutrophyc lake; the last time that a bloom occurred was in 1990-91. Phytoplankton research showed that blooms were caused by mass development of blue-green toxic algae (Nodularia spumigena and Anabaena elenkinii). They caused also massive mortality of fish (grey mullet), The accumulation of high quantities of "mucilage" was observed on the surface of water.

An extensive algal bloom occurred in Paravani Lake (eastern Georgia) in 1979-80. A large number of fish (sig, trout) was killed; cows suffered of diarrhoea after having drunk the water. Studies on phytoplankton showed that the bloom was caused by mass development of Anabaena flos-aquae and Aphanizomenon flos-aquae. Since then, algal bloom events have not been recorded in this area.

Blooms of non-toxic as well as toxic algal species were recorded in Georgia waters in different periods. Among toxic species, blooms of Microcystis aeruginosa were more frequent. although mass developments of Coelosphaerium kuertzingianum have also been found. Harmful algal bloom events were often accompanied by unpleasant smell and massive fish mortality, Cases of human poisoning related to harmful algal blooms have not been recorded in our country.

In recent years some obstacles in research on waters arose. Identification of biotoxins and studies of their action upon fish and shellfish have not been performed. Rules and laws for the control of fish contamination are now being imposed, A monitoring programme is also in process of forming. Among non-official programmes it is significant the programme of Georgian "Ecological Centre " concerning the determination of the mutagenic action of different toxins in organisms by using the method of rnetaphasic preparation.

GREECE

Marine pollution and eutrophication problems in Greece arc localized mainly in estuaries and embayments in proximity to the largest industrial and urban centres. Enrichment of the naturally oligotrophic sea water of these gulfs, effected mostly by the discharge of untreated domestic-industrial effluents, has disturbed the marine ecosystem equilibrium. The most disturbed marine areas are the Gulfs of Saronikos, Thermaikos, Pagassitikos, Amvrakikos, Alexandroupolis and Kavala (Pagou, 1990).

Blooms of Gymnodinium breve associated with fish kills have been observed only twice in Saronikos Gulf in November 1977 and October 1987 (Pagou, 1990: Pagou and Ignatiades, 1990). However, toxicological studies associated with these blooms were not carried out. Red tide phenomena have been recorded also in Kavala Gulf in August 1986, where the most abundant species was Alexandrium tamarense (Panagiotides et al., 1987) and in Pagassitikos Gulf, in July 1987, where a Gymnodinium catenatum like species was dominant (Friligos and Gotsis-Skretas, 1989).

In a research programme carried out in the Athens School of Hygiene, in the framework of Med. Pol. II, PSP toxins were monitored during 1985-1988 in samples of mussels from Elefsis Bay (Saronikos Gulf). In all the 140 samples examined, PSP was always below the detectable level of the biological method (Papadakis, 1991).

According to Council Directive 91/492/EEC, bivalves from designated areas of culture should be examined for PSP and DSP and also water zones for growth and relaying of shellfish should be monitored for toxic phytoplankton. In compliance to this Directive, all samples examined so far by the Institutes of Food Hygiene in Athens and Thessaloniki, using the biological method, had PSP <80mg/100g meat (personal communication).

Finally, incidents of poisoning associated with the consumption of toxic shellfish have never been reported in the Poison Control Centre in Athens.

In conclusion, although toxic algae problems are not significant in Greece, the application of a monitoring programme should be envisaged in the context of the global expansion of toxic blooms lately.

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GUATEMALA

A severe outbreak of Paralytic Shellfish Poisoning occurred in the village of Champerico on the Pacific coast of Guatemala in the last week of July 1987, Of 187 people affected, 26 deaths were recorded. The vector was identified as a population of small donacid clams, Amphichaena kindermani, which appeared in the surf zone of the exposed beach. The toxins accumulated by the clams were produced by a dense bloom of Pyrodinium bahamense var compressum present along the shore.

In laboratory analysis carried out in the Food and Drug Laboratory of the Ministry of Health (LUCAM) in Guatemala, using the mouse bioassay method, the highest level detected was 6000 µg saxitoxin equivalents/100 g of sample (AOAC, 1984; Unified Food and Drug Control Laboratory, 1987). These results were confirmed at the FDA Laboratory in Washington, D. C., using HPLC method (Sullivan and Iwaoka, 1983). Those results indicated that 21 -sulfosaxitoxin was the major component present in shellfish extracts. Saxitoxin and l-N-hydroxisaxitoxin (neosaxitoxin) were also detected in smaller amounts. Initially, organophosphates poisoning was suspected. This hypothesis was discarded after samples of food and stomach contents of victims were analyzed for pesticides. None of these were found (Unified Food and Drug Control Laboratory, 1987).

Previously, another documented episode of visible red tide occurred in Puerto Quetzal, also on the Pacific coast of Guatemala, in August 1985. In this case only dead finfish was reported. The responsible organism was not identified. After 1987 several red tide occurrences have been documented on the Pacific shores of Mexico, Central America and Panama (Campos and Canahui, 1992). In all cases P. bahamense was identified. Another interesting case is worth mentioning: a permanent presence of a red tide has been observed in front of the pier of Puerto Quetzal (Pacific coast of Guatemala). In this case the organism responsible was Cochlodinium polykrikoides. There is no report of fish mortality.

A Guatemala Commission for the Control of Red Tide was organized in 1987. This Commission is integrated by delegates of the Ministries of Health and Agriculture, the University of San Carlos, and the Institute of Meteorology. The activities of the group are the implementation of a monthly monitoring program, sampling at three locations along the Pacific coast of Guatemala. Shellfish samples have been examined in the laboratory using the mouse bioassay method (Association of Official Analytical Chemists, 1984).

Until now there are no ciguatera cases reported in Guatemala

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ITALY

The greatest percentage (68 %) of the Italian shellfish production is obtained in the Adriatic Sea, particularly in the Northern area, Many species are cultivated, but the production is mostly focused on Mytilus galloprovincialis and Tapes semidecussatus.

In 1989 the first case of human DSP intoxication occurred along the Emilia Romagna coasts (Adriatic Sea) (Boni et al., 1993). During the following years, DSP contamination of shellfish has been repeatedly reported along the italian Adriatic coastline from the Gulf of Trieste to the coasts of Marche (Ammazzalorso et al., 1991; Fattorusso et al., 1992; Tubaro et al., 1992; Boni et al., 1993; Della Loggia et al., 1993; Tubaro et al., 1995). Mussels (from both natural banks and farms) appeared to be the most contaminated shellfish.

Therefore, the collection and marketing of mussels in this area has been banned since 1989 for long periods with considerable economical losses. The DSP risk period occurs in summer until the beginning of fall. The main DSP toxin found in the Adriatic Sea is okadaic acid (Fattorusso et al., 1992: Zhao et al., 1994). Various Dinophysis species have been considered responsible for DSP in the Adriatic Sea: Dinophysis fortii is regarded as the main source of DSP toxicity in the Gulf of Trieste (Della Loggia et al., 1993; Tubaro et al,, 1995), while it is still uncertain which are the DSP causative agents and the role of the different Dinophysis species in the DSP episodes occurred along the other parts of the Adriatic coast (Boni et al., 1993).

In May 1994 mussel samples from the Emilia Romagna coast were found to be contaminated by PSP toxins in correspondence with a bloom of Alexandriurn minutum. The toxin profile of an A. minutum sample collected during the bloom revealed the presence of GTX-2 and GTX-3, Potentially PSP species belonging to the genus Alexandrium have been repeatedly found in the Northern Adriatic Sea since 1982. but no PSP contamination has been detected until 1994. A. minutum had already formed an offshore bloom in the Northern Adriatic Sea in 1990, but no contamination was detected probably for the distance from the coast, since the mussel farms are located near the coast. Cysts of Alexandrium spp. were also found in the sediments of the Gulf of Trieste (Northern Adriatic Sea). Pseudo-nitzschia species are frequently found in high amounts along the Adriatic italian coasts, but no ASP contamination was ever detected.

The Italian law prescribes that shellfish growing areas must be monitored by Regional Health Authorities for shellfish toxicity and the presence of toxic phytoplankton fortnightly. Seawater samples have to be collected at three different depths (0.5 m from surface, middle and bottom). If more than 1000 Dinophysis cell/1 are present, or other potentially toxic species are found, or toxicity is detected in shellfish, the sampling must be intensified.

The shellfish marketing is prohibited when the shellfish contains:

(i) a concentration of DSP toxins that gives positive results to the mouse bioassay (survival time < 5 hrs);

(ii) more than 80 µg of PSP toxins/ 100g of soft tissue, determined by the AOAC method:

(iii) a concentration of NSP toxins detectable by the Mc Farren method.

Researches are also carried out to set up a quick alerting system for shellfish contamination risk based on alternative monitoring methods: different sampling methods (bottle, net, etc.) for the rapid detection of toxic phytoplankton are tested; new methods for DSP toxins detection (such as Protein Phosphatase 2A inhibition by okadaic acid) are developed.

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KENYA

In Kenya, massive fish mortalities occur occasionally in Lake Victoria under specific and sporadic climatic conditions, which lead to the deoxygenation of the water column. In the main lake, deoxygenation has been shown to occur regularly in deep water under strong stratification (Fish, 1957; Tailing, 1966). The Nyanza Gulf of Lake Victoria is generally shallow and thus heavy storms can stir up the bottom sediment and distribute it throughout the the water column. This organic mud added to the nutrients from the storm run-off and the increase in pollution can support algal blooms which eventually lead to oxygen deficiencies.

Nile Perch (Lates niloticus) and tilapia (Oreochromis niloticus) form the bulk of the fisheries catch at present in the Nyanza Gulf (Kenya Govt. Rep., 1983). In late August through October 1984, large numbers of both species were found dead along the shores of the Nyanza Gulf. Low pH, low dissolved oxygen concentration, clogged gills with algal and sediment floes appear to be the main causative factors in the fish mortality. Each of these components was attributed to the presence of unusually dense algal blooms of Anabaena flos-aquae and their subsequent breakdown during the fall of 1984. A previous study

in the Nyanza Gulf (Melack, 1979) revealed that among other cyanophytes, Arzabaena sp. dominates during the period of August-December.

Between February and August 1986, another toxic algal bloom was observed in the open waters of Lake Victoria. Mycrosystis aeriginosa accounted for > 90% of the bloom organisms with algal counts reaching a high density of 34,000 colonies per ml. The release of nutrients from river inflows, from upwelling and from sediments into the euphotic zone, coupled with high temperatures, produced the observed blooms. The blooms subsequently declined as a result of physical flushing, temperature reduction associated with the rainy season and nutrient depleting. In the period 1991/1992 a major algal bloom of the Mycrosystis sp. occurred at the salt farms along the Kenyan coast causing fish kills and 70% loss in production. Following this infestation, Artemia (from San Francisco Bay) (SFB) was introduced into the saltfarmsand it successfully controlled the algal bloom.

Lately, routine monitoring by Kenya Marine & Fisheries Research Institute has reported frequent occurrences of non-toxic algal blooms, both in the Kenya waters of Lake Victoria and the Gazi creek at the coast, where oyster farming is in progress.

MOROCCO

Fish and shellfish are harvested along the Atlantic and Mediterranean Moroccan coasts. While oysters (Crassostrea gigas and Ostrea edulis) and carpet-shell clams (Ruditapes decussatus) are cultivated at an industrial scale, mussels (Mytilus galloprovincialis and Perna picta) are heavily fished from natural beds.

The phenomenon of red tides has been certainly known for a long time in Morocco, but only in the late 1960's it attracted the awarness of local authorities. Several red tides were registered in both the Atlantic Ocean and in the Mediterranean Sea. It seems that red tides occur regularly in a cyclic manner. There are two period of risk: mainly late summer and early autumn (September-November) and, to a lesser degree, between January and March. To date, there have been two significant outbreaks of shellfish poisoning. The first one occurred in 1971 and involved 114 victims, of which 9 died. The second one occurred recently in November 1994 and involved 70 cases with 4 casualties. These two outbreaks occurred after an excessive growth of dinoflagellates. Mussels clandestinely harvested from areas closed for shellfishing where the vector in those outbreaks.

Red tides were mainly caused by excessive growth of dinoflagellates and diatoms. In particular the genera involved were Peridinium, Gymnodinium and Noctiluca. According to epidemiological data, field surveys, clinical symptoms and laboratory analysis, saxitoxin was the phycotoxin involved in the deadly outbreaks of PSP. In laboratory analysis, attention is mainly paid to saxitoxin: it is thus difficult to determine whether other toxins have been involved in some outbreaks of intoxication. The shellfish contaminated and/or responsible for poisoning were mussels, oysters, cockles, clams, Venus and Donax.

In Morocco, there is a control system for the quality-assurance of finfish and shellfish because seafood is an important source of employment and foreign exchange. Almost 80% of Moroccan seafood is exported mainly to Europe. For the need of foreign exchange, the rules and guidelines are mainly based on those of the EC (Guidelines 91/492/CEE and 91/493/CEE). Before export, finfish is subject to chemical and microbiological analyses. Official laboratories agreed by the EC are in charge of these analyses. In many plants, quality-assurance of finfish (fresh, frozen, canned) is based on the HACCP concept (Hazard Analysis of Critical Control Points).

For shellfish, there is a Moroccan legislation related to the control of salubrity of shellfish. This legislation involves the determination of rules, concerning:

(i) delimitation and classification of shellfish growing waters. The classification is the following: approved or open areas, suspected areas and closed areas. A list of these areas is available and it is communicated to the 'EC. For the sake and safety of the public, suspected and closed areas are clearly identified by panels;
(ii) surveillance of shellfish growing areas and shellfish plants;

(iii) transportation and packaging of shellfish;

(iv) qualitative and sanitary inspection of shellfish, shellfish growing area, retaile-market, deputation plants and frontiers.

Surveillance and laboratory analyses of shellfish and shellfish growing area are performed by Official Veterinary Laboratories (Ministry of Agriculture), Ministry of Fisheries and Ministry of Public Health (National Institute of Hygene).

The EC Directive 93/387/CEE mandates the Ministry of Agriculture (Direction de l'Elevage) as the competent authority for the sanitary inspection of shellfish. According to the Moroccan legislation, fresh marketed shellfish is systematically subject to the determination of algal toxins. Analyses performed in this respect are based on the determination of saxitoxin (PSP) by the Mouse Bioassay. The guideline is 4 MU.

Recently, the national network for the monitoring and surveillance of marine waters have been set up. Several stations in the Atlantic and Mediterranean coasts are in charge of the regular determination of algal concentration in seawater and concentration of toxins in shellfish.

In case of a red tide or of an excessive concentration of algae in seawater (determined by the surveillance network), there is an alert and the public is immediately informed. There is also a ban on shellfish harvesting and selling with a stricter control on clandestine harvesting of shellfish. There is also an Anti-Poison National Center for the public to obtain information about these events.

It is reasonable to assume that, in the next future, the industry of aquiculture will flourish in Morocco. Efforts are presently made for the monitoring and surveillance of coastal waters in order to prevent dramatic events such as those occured in 1971 and 1994.

ROMANIA

It is known that, as a result of human activities (industrialization, agriculture, fertilization, urbanization) in all the countries of the Danube basin, the content in nutrients and organic matter in the seawater increased. This high concentration of nutrients in the seawater caused ecological consequences such as the increase in frequency and intensity of phytoplankton bloom. From 1981 to 1994, 65 blooms were produced, 17 of them having tremendous development (the concentration of the species involved was more than 50 millions cells per liter) and 18 algal species, belonging to different systematic groups, have been implicated. The most frequent ones were: Prorocentrum cordatum, Heterocapsa triquetra, Eutreptia lanowii, Emiliania huxley, Skeletonema costatum, Cerataulina pelagica. The differences in number and the entity of blooms depended on the seasonal hydrobiological peculiarities of the Black Sea.

The blooms produced by the microalgae represent an important reason of disturbance in the ecosystem determining an excessive concentration of organic matter and a depletion of oxygen content. The hypoxia, anoxia and the massive mortality of benthic fauna which follow the blooms, have induced a deterioration in biological conditions as well as a decrease in the abundance of both invertebrate and fish, including commercial stocks. Furthermore, the eutrophication may represent a favourable environment for the establishment of some algae introduced in the Black Sea, for example by ships' ballast water.

The increase of bloom frequency and their undesirable ecological and socio-economical consequences have imposed the necessity of investigations in the framework of our research programmes. Since 1983, a planned ecological study and the monitoring of algal blooms have been organized at Romanian Marine Research Institute. At present, this study is a part of the programme for a better knowledge of the marine ecosystem state and the evaluation of the eutrophication impact. In this framework the researches are focused on:

(i) phytoplanktonic quantitative and qualitative dynamics;

(ii) the evolution of the blooms, based on the comparison between previous and recent data;

(iii) the micromicete population dynamics retaled to the entity of algal blooms;

(iv) the correlation with salinity, temperature, pH, nitrate, nitrite, ammonium, phosphate content and with the main biochemical compounds (proteins, lipids, carbohydrates and nucleic acid) in the seawater;

(v) the evaluation of ecological effects of algal blooms.

The species that caused blooms more frequently, along the Romanian Black Sea literal, were Prorocentrum cordatum, Skeletonema costatum and Eutreptia launowii; 9 other species produced such phenomena sporadically (Bodeanu, 1995).

In addition to the activity developed in this National Program, which is financed by the Romanian Ministry of Research and Technology, our Institute cooperates in the achievement of some regional projects for ecological researches in the Black Sea, such as:

(i) NATO (OTAN) - TU Black Sea interested in eutrophication phenomena when these concern the inferior trophic level;

(ii) the European Community Project EROS 2000, also with problems related to eutrophication and contaminants;

(iii) the COMS Black Program in which it is emphasised that the eutrophication is the principal problem of the marine environment.

None of these programmes concerns the problem of Toxic Algal Blooms, therefore we do not have any information about algae or shellfish involved in toxic events.

The Romanian legislation, related to the hygiene-sanitary conditions of food products, do not include toxicological regulamentations for marine shellfish concerning the algal toxins. On the other hand, there is not a significant industry related to the mussel in the Black Sea and their consumption by the local population is limited. If the use of mussels as a food source will be introduced in Romania in the future, as it seems probable, the sanitary law must be completely based on the researches and the data already performed.

Ecological, sanitary and scientific reasons will make necessary a Harmful Algal Blooms programme in Romania.

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RUSSIA

A group for harmful algae research has been formed in 1995 at P. P. Shirshov Institute of Oceanology, Russian Academy of Sciences, Moscow, as a result of the growing awareness of the risks brought about by harmful algal blooms. Russian National Programme on Research and Monitoring of Harmful Algal Blooms is being promoted by the Ministry of Science and Technical Politics of Russian Federation

No systematic research of harmful algae in Russian seas has been performed as yet. Nevertheless we have a substantial amount of reports on visual observations of algal blooms in Baltic Sea (Kurshsky

Gulf), White sea (Dvinsky Gulf), Black Sea (Gelendjik Bay), Japan Sea (near Vladivostok) and at the Pacific coast of Kamchatka; the nature of the blooms is poorly investigated. The only well documented harmful algal event was described in 1988 in Amursky Gulf (Japan sea) when the peak cell concentration of Chattonella sp. reached $15x10^6$ cells/1. Also dangerous densities (tens of thousands cells/1) of several toxic Dinophysis species occur every summer at the Russian coast of Japan sea.

No medical statistics on algal toxin poisoning exists in Russia and it is unknown whether this situation is caused by the actual absence of such cases or by misdiagnostics. There are no legal acts regulating the control of seafood for the presence of algal toxins in Russia. It must be noted that shellfish represent a traditional part of diet only for the population of the Japan Sea coast, and to less extent of the Black Sea coast. Nevertheless scallop meat from Far-Eastern Russia is being sold in food shops and it is served in restaurants in many Russian cities.

The role of marine aquiculture on Russian fish market is negligible at the present time, so the possible impact of harmful algal blooms on this branch of industry should be minimal; it will grow with the expected development of aquiculture.

SLOVENIA

In October 1984, an unusual green dinoflagellate bloom appeared in the waters of the Gulf of Trieste. The bloom was monospecific and due to Gymnodinium sp. The consumers of mussels claimed gastrointestinal disorders and therefore the mouse bioassay was performed to test the possible toxicity of fat-soluble and water-soluble extracts from the mussel tissue and dinoflagellate samples. The samples were non-toxic; however, shellfish samples of various origins were routinely tested for the presence of biotoxins in our institute because of the mentioned event. In September 1989, we established the presence of Dinophysis spp. in water samples originating from the northern Adriatic. All shellfish on the market were immediately tested for the presence of toxins. The specimens grown in the northern Adriatic were toxic, whereas those from the middle Adriatic were not toxic (Sedmak et al., 1990). The positive mouse tests resulted in the prohibition of sale of shellfish from the two major shellfish farms in Slovenia. The farm in Seca was closed down for two months until December 1989 and the farm in Strunjan for four months until January 1990. The shellfish market in Slovenia depends heavily on export to the Italian market. In the years following the attainment of independence the Slovene shellfish market collapsed as a result of bureaucratic disagreements with Italy.

Nevertheless, a monitoring program was introduced to protect human health. Currently it includes monitoring phytoplankton populations with a special reference to the potentially toxic dinoflagellates Dinophysis spp. and Alexandrium spp. and bioassays on tissue extracts of shellfish.

The phytoplankton sampling frequency varies according to the seasonal abundance of the harmful species of interest. It starts in June and lasts at least until the beginning of October. The presence of the potentially toxic genera Dinophysis or Alexandrium in the water stimulates to a more frequent sampling program. Net hauls, analyzed for suspected dinoflagellates, had revealed the presence of different species of Dinophysis (D. fortii and D. acuminata were the most frequent) (Sedmak and Fanuko, 1991).

In order to examine the safety of shellfish, the mouse bioassay was used as the method authorized by Slovene authorities. Different shellfish species were tested for the presence of DST, PST and NST. The mouse bioassay for PSP and NSP was performed according to the AOAC standards. Diarrhoeic shellfish toxins have been routinely detected using a mouse test based on the methodology developed in Japan (Yasumoto et al., 1978). In the last two years, the survival time of mice, which is required to establish whether shellfish represents a risk to consumers, has been reduced to 5 hours. This method has proven very useful in the field, since under this regulatory limit, no cases of poisoning have been established in France (Marcaillou-Le Baut et al., 1994). The same limit has been selected as a criterion for shellfish prohibition also in Italy (G. U., 1990). In its efforts to join the EC, Slovenia is adopting the relevant EC legislation, although it has already a law prohibiting biotoxin contents in food (Ur. 1. n.68/89).

In the last two years DSP toxins have been detected by bioassay in the summer and autumn, resulting in the closure of affected areas to harvesting. The following temporary bans on taking shellfish

have been imposed on Slovene shellfish farms: in 1993 from October 11 untill November 22. and in 1994 from August 9 untill September 27.

Besides the cultivated mussel (Mytilus galloprovincialis Lam.) all shellfish from the fish market and various wild specimens were tested for toxicity. In 1994 an additional program of monitoring toxic cyanobacteria (blue-green algae) started (Sedmak et al., 1994).

According to the results of our investigations we can confirm that so far the North Adriatic sea is a safe area concerning neurotoxic shellfish poisoning (NSP), Thus is not the case of diarrhoeic shellfish poisoning (DSP). We can not exclude PSP outbreaks in the future, There is also the possibility of the presence of protein phosphatase inhibitors of the microcystin class in the marine environment (Chen et al., 1993).

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SPAIN

The first reported toxic episode took place in 1976, when PSP occurred in various European countries caused by mussels from Galicia. The responsible organism was later identified as Gymnodinium catenatum. The first cases of gastroenteritis associated with DSP were in 1981. and affected 5000 people. Following the toxic PSP event in 1976, monitoring and control programmes of potential toxic algae. shellfish and environmental parameters were established in the areas involved in shellfish production. The main production area is the Galician Rias (NW of Spain), that are the world producers of raft cultivated blue mussels, followed by the Ebro Delta region (Northern Mediterranean coast), Andalusia (South) and Valencia (East). Although their lower production, they have also implemented surveillance programmes. since several toxic and non toxic phytoplankton blooms have been observed in the last years.

PSP is associated with Alexandriurn rninutum and Gymnodinium catenatum. Concerning toxin profiles, A. minutum is rich in carbamoyl toxins such as GTX 1-4 and G. catenatum mainly produces sulfocarbamoyl toxins. Dinophysis acuta and D. acuminata have been associated with DSP outbreaks, Okadaic acid is the predominant DSP toxin although DTX-2 has been recorded in shellfish and

phytoplankton and acyl-derivatives of okadaic acid have recently been found in Galician mussels. Several strains of Prorocentrum lima isolated from Galician waters produce DSP toxins as okadaic acid and DTX-1. Although this benthic dinoflagellate grows on macroalgae on mussel ropes, its implication in mussel intoxication has not been demonstrated. Domoic acid in mussels at concentration slightly below the tolerance level established in Canada followed a bloom of Pseudo-nitzschia australis in October 1994.

The European Council Directives 91/491 and 91/492, laying down the requirements for the extraction, production and placing in the market of live bivalves and fishery products, have been transposed to the national regulations and the guidelines followed for the sanitary control of seafoods.

From 1977 to 1992, the Spanish Oceanographic Institute (IEO), belonging to the Agriculture Ministry was in charge of the water monitoring. Since 1992, the health departments of the regional governments are responsible for water and shellfish surveillance, always in close collaboration with the IEO. The Central Health Department (Ministerio de Sanidad y Consumo) is in charge of the seafood safety for import-export and its laboratory in Vigo was nominated European Community Reference Laboratory on marine biotoxins in 1994.

The Galician monitoring programme is based on a systematic sampling of the production sites. Water sampling is made weekly during the whole year. Each Ria (oceanic bay), is divided into different sampling zones and following physical, chemical and biological parameters are monitored. Temperature, salinity, pH, dissolved O_2, photosynthetic pigments, nutrients and cell counts of phytoplanktonic populations. Regarding shellfish monitoring, the production areas are also divided into different zones which have several sampling sites. In the case of raft cultured mussels, there are sixty sampling sites, and samples are collected from two opposite ropes at three different depths. Species involved in toxic episodes that are currently controlled include bivalves as mussels, clams, scallops, cockles, littlenecks and oysters; crustaceans and gastropodes such as ormers.

The method employed for PSP detection with regulatory purposes is the AOAC Mouse Bioassay (Hollinworht and Wekell, 1990). DSP is monitored by the Yasumoto Mouse Bioassay (Yasumoto et al., 1978). Both assays are supported with HPLC techniques. The ASP testing procedures is that of Lawrence et al. (1991).

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URUGUAY

In 1980 intoxication problems due to the consumption of molluscs (Mytilus edulis) were registered in Uruguay for the first time. Since then we established a monitoring programme that comprises different stations along the ocean coast. The austral situation of the country, the characteristics of the ocean coast and the main harbour, could be some factors to be considered in the development of the toxicity phenomena in our country. Since 1991 an increment in the phenomena frequency is observed, registering 1-2 events per year, although no human toxicity cases were detected.

Red tide phenomena have increased around the world and Uruguay does not run away from this situation. They are the result of plankton toxic dinoflagellates proliferation. Due to the fact that they may

course without water discoloration, actually it is better to talk about Harmful Algae Bloom's, Toxins elaborated by this dinoflagellates affect public health and sometimes may cause human death.

Many factors could be responsible of these phenomena in Uruguay:

(i) The southern geographic localization of the country: some authors have seen changes in the phytoplanktonic communities probably due to ozone layers slenderness.

(ii) Uruguay has Atlantic Oceans coasts that conforms a confluence zone: a northward current and a southward cold current coming from an estuary that receives the Rio de la Plata river nutrients. This represents an hydrological changing front, with salinity discontinuation, current convergence. etc.

(iii) The main harbour has an important shipment traffic, with ballast water discharges that may be responsible of the sediment cyst translation,

The first human toxicity cases were registered in 1980 with typical PSP symptoms, although no human deaths occurred. Those episodes were the consequence of Gonyaulax sp. proliferation (actually identified as Alexandrium fraterculus), reaching the maximum value of 1.800 mg saxitoxin equivalents/ 100 g. Acidified samples were sent to Dr. Y. Shimizu of the Rhode Island University, who reported the presence of saxitoxine, neo-saxitoxine and gonyaultoxin I, II, III and IV.

Since then, we established a monitoring programme along the ocean coast with samples taken both from easy area accessible also to vacationists, and from mussel farmers near the coast. The programme includes water (in order to investigate the phytoplanktonic communities, salinity, temperature and level tide registers, etc.) and molluscs monitoring: Mytilus edulis, Mesodesma mactroides, Donax hanleyanus and Chlamys sp. The work is done by a multidisciplinary team, weekly in summer, and fortnightly or monthly in other seasons.

In 1991, in coincidence with an abnormal rise of the temperature for that season, we registered a bloom whose maximum toxicity value was 8.300 g/100 g, Alexandrium excavatum was the organism responsible. The HPLC analysis in Vigo, revealed the presence of GTX 3, Other samples were sent to Dr. S. Hall of the FDA. Since then, we have had one or two blooms per year, which is coincident with frequency world increment. In 1992, due to extraofficial advices referred to diarrhetical episodes. we began to investigate the DSP toxins in bivalve molluscs, in coincidence with the presence of Dinophysis acuminata in water samples.

To detect PSP toxins the AOAC Mouse Bioassay is used, in order to obtain an acid extract that is inoculated in mice of approximately 21 g so as to determine the death time and the mg saxitoxin equivalents/ 100 g of the sample. It is a quantitative technique and safety level for human consumption in our country is 80 mg/100 g. For the DSP toxins detection, a mouse bioassay modified by Yasumoto, which consists of acetone extraction and intraperitoneal inoculation in mice of 21 g weight, was used. If mice death occurs in 24 hours, it is considered a positive sample.

When hazard levels to human consumption are detected for each toxin, the Ministry of Livestock, Agriculture and Fisheries prohibited to collect, consume and commercialize bivalve molluscs. and the Ministry of Public Health is notified.

From the toxicological point of view, we intend to continue with the Mouse Bioassay and will try to set up the HPLC technique and others available methods.

Years	PSP			DSP		
	n°of samples	n°of positives	%	n° of samples	n° of positives	%
1980*	117	50	43			
1991	162	39	24			
1992	231	23	10	27	2	7
1993	275	55	20	5	0	0
1994	200	3	1,5	7	1	14,3

* From 1981 to 1990 the toxin monitoring continued, with negative results

ANNEX IV

ROUND TABLE ON NATIONAL REGULATIONS FOR MARINE PHYCOTOXINS

A Round Table on the current national regulations for the control of marine phycotoxins in food has been held, in order to examine the regulatory situation in the countries represented at the Course. All the lecturers and the participants to the course have been involved in the discussion. The situation in the following 15 countries was considered: Albania, Austria, Croatia, Georgia, Greece, Guatemala, Italy, Japan, Kenya, Morocco, Romania, Russia, Slovenia, Spain and Uruguay.

As shown in Table I, only two of the countries considered possess official regulation for PSP, DSP and NSP, whereas seven countries regulate the control of PSP and DSP, two countries control only PSP and six countries do not perform any official control on phycotoxins.

Table I. Regulatory situation on phycotoxins in the countries represented at the course

Country	PSP	DSP	NSP
Albania	no	no	no
Austria	yes	yes	no
Croatia	no	no	no
Georgia	no	no	no
Greece	yes	yes	no
Guatemala	yes	no	no
Italy	yes	yes	yes
Japan	yes	yes	no
Kenya	no	no	no
Morocco	yes	no	no
Romania	no	no	no
Russia	no	no	no
Slovenia	yes	yes	no
Spain	yes	yes	yes
Uruguay	yes	yes	no

NSP contamination is controlled in Italy by means of the McFarren method and shellfish are allowed for human consumption if no toxin presence is detected by this method. In Spain the Mouse APHA (1970) is used with a tolerance level of 10 MU/100g of meat (only imported products are controlled).

The tolerance levels and the methods used for the control of PSP and DSP are reported in Table II.

Table II. Methods and Tolerance levels for PSP and DSP

Country	PSP		DSP	
	method	tolerance levels	method	tolerance levels
Austria	HPLC[1]	40 µg/100 g	HPLC	OA not detectable
Greece	AOAC[2]	80 µg/100 g	MB[5]	OA not detectable
Guatemala	AOAC[3]	80 µg/100 g	not controlled	
Italy	AOAC	80 µg/100 g	MB	survival > 5 hours[6]
Japan	AOAC[4]	4 MU/g	MB	5 MU/100 g meat (20 µg OA /100 g meat)
Morocco	AOAC	4 MU/g (80 µg/100 g for export)	not controlled	
Slovenia	AOAC	80 µg/100 g	MB	survival > 5 hours
Spain	AOAC HPLC	80 µg/100 g	MB HPLC	survival > 24 hours[7]
Uruguay	AOAC	80 µg/100 g	MB	survival > 24 hours

[1] as modified by Lawrence; [2]AOAC = Mouse Bioassay by the Association Official Analytical Chemists; [3] with 1.0N HC1; [4] without mice calibration on STX; [5] MB = Mouse Bioassay by Yasumoto; [6] mean value of 3 animals; [7] median value of 3 animals.

The Authorities responsible for the control of the contamination level by phycotoxins in food are different in the various countries as reported in Table III.

Table III Responsible Authorities for the Phycotoxin Control

Country	Authority
Austria	Ministry of Health and Consumer Protection
Greece	Ministry of Agriculture - Institute of Food Hygiene
Guatemala	Ministry of Agriculture & Ministry of Public Health
Italy	Regional Health Authority
Japan	Ministry of Health and Welfare
Morocco	Min. of Agriculture & Min. of Public Health & Min. of Fisheries
Slovenia	National Veterinary Service
Spain	Regional Health Authority (Ministry of Health: for import-export)
Uruguay	Ministry of Livestock, Agriculture and Fisheries

Although the picture emerging from the comparison of the procedures used in these 15 countries is not representative for the regulatory situation all around the world, some general consideration can be proposed.

First of all, a number of countries apparently do not perform any control on the contamination level of phycotoxins. This may be due to a low relevance of the shellfish production in these countries. However, the shellfish production may represent an interesting economical resource, for instance for developing countries, both for local use and for export. However, in order to exploit this resource, a reliable monitoring regulation in the field of the phycotoxins contamination is required.

Among the countries that possess regulation in the field of phycotoxins, differences in the kind of monitored phycotoxins is observed. All the countries control the PSP and - except Guatemala - DSP. but only two control NSP and only one controls ASP. This may be due to the actual absence of NSP or ASP phenomena in these countries. However, the possibility of changes in the toxin-producing organisms pattern in the coastal regions of these countries as well as the import problems should be taken into account.

The official methods used in the considered countries to determine the contamination level are quite homogeneous: Mouse Bioassays are used for both PSP and DSP in all countries, except Austria where the use of animals is not allowed for regulatory controls. The safety thresholds are quite homogeneous for PSP, but differences are observed in the case of DSP: Spain, Uruguay and Japan take a survival time shorter than 24 hours as an endpoint for the presence of a non acceptable DSP contamination level. Italy and Slovenia take an endpoint of 5 hours, whereas Greece and Austria requires “not detectable” amounts of okadaic acid.

The differences in the endpoints for DSP may be related to differences in the toxin profile. Indeed, if acil-derivatives of okadaic acid are present, a longer time is required for the metabolic hydrolysis to release the unsubstitued toxin, that is the effective toxic agent.

Among the considered countries, a high variability in the responsible authorities is noted and in some cases an overlapping of jurisdiction is seen.

From this analysis of the regulatory situation in the countries represented at the Round Table, the need of a certain degree of standardization is evident. First of all, the participants agree upon the need for setting up a systematical monitoring of the phycotoxin contamination by all the countries that, at present, do not possess any regulation in the field, but has (or are planning) shellfish production. This requires an adequate scientific and technologic level, that can be secured only by the presence in each country of research groups involved in the studies of the phenomenon of phycotoxins from different points of view. The role of the present IOC-Course is pivotal in order to stimulate the establishment of such kind of research groups in countries where they are so far absent.

The monitoring activity has to be based upon a definite legislation. This poses the question about the needed degree of standardization among the regulation in the different countries. The participants agreed that, in the routine controls, the kind of toxins to be controlled and the methods to be used have to take into account the particular situation of each country (toxin profile usually present in local shellfish, technological level and resources at disposition etc.). However, since the actual situation may change, a more general monitoring activity should be performed by specialized research groups (not necessarily involved in the official routine controls) in order to screen for potential the appearance of new toxins and to evaluate the opportunity of changes in the official methodology.

On the contrary, the safety thresholds for the different toxins should be strictly standardized all over the world, on the basis of the present knowledge about the actual toxicogenic risk of each group of toxins.

The participants agreed that a general requirement for a good national regulation in the field of phycotoxins is the presence of a legislative procedure allowing the adjustment of the regulation to the scientific and technological progress and to the evolution of harmful algae bloom phenomenon.

Last but not least, overlapping of jurisdiction should be avoided, since it can bring to conflicting policies in managing the involved activities. For the management of the activities involved in the phycotoxin contamination control, a central authority may be suitable for countries with limited coastal areas or for countries with a well organized and developed monitoring net. In other situations, local or regional authorities, in direct contact with the production areas may probably better manage the actual problems in the area.

ANNEX V

ABSTRACTS OF LECTURES

DIFFERENT KINDS OF POISONINGS DUE TO THE SHELLFISH INGESTION (Dr. Aurelia Tubaro)

Bivalve shellfish feed by filtering water: if a toxic dinoflagellate bloom occurs, the shellfish of that area is likely to serve as unwitting concentrator of the algal toxins. Human ingestion of shellfish contaminated with microalgal toxins causes various types of poisonings, depending on the algae involved. Shellfish contaminated by dinoflagellates can provoke PSP, DSP and NSP. Dinoflagellates are also responsible for another poisoning, CFP, in which fish, living mainly in the coral reef, act as transvector. On the contrary, ASP is an intoxication due to the ingestion of shellfish contaminated by diatoms.

The phycotoxins responsible for these poisonings are generally resistant to the processing and heat treatments, that are applied to shellfish to make it suitable for consumption.

PSP is one of the most serious intoxication: it is caused by the consumption of shellfish contaminated by saxitoxin and its derivatives. These kind of toxins are readily absorbed through the gastrointestinal mucosa and the poisoned patients developed quickly neurosensorial symptoms as well as neuromuscular symptoms. The cause of death is asphyxiation, due to progressive respiratory muscle paralysis, and death can be avoided by artificial respiration. No specific antidotes are presently available. The mechanism of action of saxitoxin is a selective block of the voltage-gated sodium channels of nerves, muscle and other excitable membranes. A diagnosis of PSP is based chiefly on a history of ingestion of contaminated seafood, shortly before the characteristic symptoms become manifest. Sometimes patients presenting similar symptoms require a differential diagnosis, as on the poisonings caused by anticholinesterase pesticides, intoxications due to the ingestion of puffer fish and Botulinum poisoning. A specific receptor assay for the detection of PSP toxins in blood and urine of the poisoned patients seems to be very promising in this respect (Van Dolah et al. 1995).

DSP is a gastrointestinal disease due to the ingestion of shellfish contaminated by dinoflagellates. Since the gastrointestinal symptoms can be easily attributed to a microbial contamination of seafood, the actual incidence of DSP could be underestimated. The main symptom is diarrhoea characterized by watery faeces: some patients can develop also nausea, vomiting and abdominal pain. Normally the victims recover in few days, despite of the received treatment. In any case, no specific antidote is available and only a symptomatic treatment can be supplied. The diagnosis is made on the basis of the history of the patient: no specific laboratory tests are presently available.

OA, the main DSP toxin, is a potent and selective inhibitor of Protein Phosphatase 1 and 2A. The toxin probably causes diarrhoea by stimulating the phosphorylation of proteins that control sodium secretion in intestinal cells or by enhancing phosphorylation of cytoskeletal or junctional elements which regulate the permeability to solutes, thereby resulting in a passive loss of fluids (Aune and Yndestad, 1993). OA and DTX-1 develop a potent tumor promotor activity on mice and rats, but their long term effects on human beings are not yet fully elucidated.

In Canada, in late 1987, there was an outbreak of a new shellfish poisoning characterized by gastrointestinal symptoms and unusual neurological abnormalities. The most peculiar symptom was the loss of short-term memory and, for this reason, the poisoning was called ASP. DA. an excitatory amino acid, was identified as the toxin responsible for ASP both in contaminated mussels and in the causative diatoms. Generally, after consumption of DA contaminated seafood, gastrointestinal symptoms, such as nausea, vomiting, diarrhoea and abdominal cramps have been observed within 24 hours. Furthermore, at least one of the following neurological symptoms or signs have been observed within 48 hours: confusion, memory

loss, disorientation, and/or seizures or coma. At present, therapy is limited to life support, but experimental work suggests that diazepam could be useful to control the seizures. The excitatory activity of DA is due to its agonistic effect on kainate receptors.

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TOXIC MARINE MICROALGAE: A GENERAL OVERVIEW (Prof. Giorgio Honsell)

Marine toxic microalgae constitute less than 2% of the world ocean flora that includes about 4000 species (Sournia, 1995): more than fifty dinoflagellates have the capacity to produce toxins, but toxic species can be found also among diatoms, prymnesiophytes, raphydophytes and cyanobacteria. Some toxic species (e.g. Dinophysis spp., Alexandrium spp. Gambierdiscus toxicus, Pseudo-nitzschia multiseries, etc.) are responsible of intoxications (PSP, NSP, DSP, ciguatera, ASP) in man and marine animals (seabirds, marine mammals) through the accumulation of their toxins in vector organisms (generally shellfish or fish). Other species (e.g. Chrysochromulina polyepis, Gymnodinium mikimotoi, Heterosigma akashiwo, etc.) can produce and release toxins directly in seawater causing kills of fish and/or other marine fauna with relevant economical losses to aquiculture.

Different authors have remarked a global increasing of toxic events related to microalgae during the last twenty years (Anderson, 1989; Smayda, 1990; Hallegraeff, 1993; Wyatt, 1995). The following points should be emphasized: 1) the number of species recognized to be toxic has been rapidly increasing, doubling in about ten years; species considered harmless have unexpectedly caused heavy intoxications (e.g. Pseudo-nitzschia multiseries and ASP) or massive fish kills (see the case of Chrysochromulina polylepis blooms); new toxic species have been described for the improvement of the taxonomic knowledge of microalgae; 2) the geographic distribution of harmful species and related toxic events has been revealing a wider extension involving new geographical areas (e.g. PSP was distributed only in the Northern emisphere until Seventies; no toxicity was detected in the Mediterranean Sea until 1987); 3) the frequency of toxic blooms has been increasing. Different kinds of factors have been related to this general trend of intensification of toxic events: the expansion of aquiculture in new areas with resident toxic species; anthropogenic eutrophication in coastal waters with nutrient enrichment and increase of plankton blooms; the long distance transport of toxic species by ships ballast waters; the enhancement of research on toxins and toxic species.

DSP is caused by different dinoflagellates belonging to the genus Dinophysis (Lee et al., 1989; Steidinger, 1993). DSP toxins have been found also in some benthic Prorocentrum species (P. lima, P. maculosum and P. hoffmannianum), but their involvement in DSP shellfish contamination needs still to be clarified. The first species involved in this intoxication was Dinophysis fortii identified as the DSP causative agent in Japan in 1978. Since then, other Dinophysis species were found to produce DSP toxins (D. acuminata, D. acuta, D. norvegica, D. mitra, D. tripos and D. rotundata) or were correlated with DSP episodes (D. sacculus and D. caudata).

PSP is caused by various dinoflagellates (several Alexandrium species, Gymnodinium catenatum and Pyrodinium bahamense var. compressum) (Steidinger, 1993). The first organism involved in this intoxication was a species now belonging to the genus Alexandrium: A. catenella. At the end of Sixties the production of PSP toxins by Alexandrium catenella and A. tamarense was demonstrated. Since then, other Alexandrium species were recognized as PSP toxins producers: Alexandrium acatenella, A.angustitabulatum, A. cf. cohorticula (?), A. tamiyavanichi, A. fraterculus, A.fundyense, A.minutum (= A.lusitanicum), A.ostenfeldii, A.tamarense (= A.excavatum). The taxonomy of the genus Alexandrium has been recently revised (Balech, 1995). Gymnodinium catenatum was associated with PSP only at the end

of Seventies, Pyrodinium bahamense var. compressum has been causing PSP in the tropical-subtropical Pacific since mid-Seventies.

ASP causative agents are some diatoms belonging to the genus Pseudo-nitzschia, although domoic acid has been found also in other two diatom genera. Pseudo-nitzschia multiseries (= Nitzshia pungens f. multiseries) was the first species involved in this intoxication. Other Pseudo-nitzschia species (Pseudo-nitzschia australis, Pseudo-nitzschia seriata, Pseudo-nitzschia delicatissirna and Pseudo-nitzschia pseudodelicatissima have then been found to produce domoic acid. In addition Also Amphora coffaeformis and Nitzschia actydrophila are reported as domoic acid producers.

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ANALYTICAL METHODS FOR DETERMINATION OF DSP AND OTHER TOXINS IN SHELLFISH, ALGAE AND SEAWATER (Prof. Takeshi Yasumoto)

Cases of poisoning due to shellfish contaminated with marine toxins are increasing all over the world and pose serious problems both to public health and to aquiculture. Although conventional mouse bio-assay methods are useful for assessing most of the responsible toxins, alternative highly specific analytical methods are required.

To assess the toxicological risk associated to DSP contamination various methods were developed in particular to determine the presence of the main DSP toxins, OA and its derivatives DTX-1 through DTX-3. In this respect, LC methods and ELISA assays are highly promising, while cytotoxicity and enzyme inhibition assays, although promising, needs further refinements. In particular, due to its sensitivity, the enzyme inhibition assay could be used to detect traces of OA also in seawater. The detection of the presence of other toxins isolated from DSP contaminated shellfish, such as PTXs and YTX appears to be more problematic for the lack of commercially available standards. Furthermore, only some of PTXs can be easily detected by LC. To overcome this problem, a monoclinal antibody, using PTX-6

as hapten, has been prepared. Since the antibody does not cross-react with other polyether toxins, it would be useful to test the presence of PTXs in shellfish, together with the fluorometric LC method. The LC assessment of YTX still faces a problem because of the laboriose clean-up procedures.

The recent outbreaks of NSP in New Zealand suggest the need of a re-evaluation of testing methods to assess the NSP risk. Moreover the definition of the true toxins responsible for NSP is a primary requirement to develop adequate analytical methods. Presently, none of the current analytical methods (LC, Binding assay etc.) used for marine phycotoxins seems to be satisfactory, if used alone.

THE MOUSE BIOASSAY FOR THE DETECTION OF DSP TOXINS (Prof. Roberto Della Loggia)

The Mouse Bioassay developed by Yasumoto is the test mostly used by health authorities for the control of the DSP contamination in shellfish. The test consists in injecting intraperitoneally some mice (usually three) with a lipophilic extract of the digestive glands of the shellfish under examination. The survival time is taken as an end point for the evaluation of the presence of DSP toxins. The test was originally developed for the detection of the toxin(s) containing fractions during the studies for the isolation of okadaic acid, and not for the quantitative determination of the toxin in shellfish.

There is no direct connection between the survival time and the diarrhoeic effect in human beings after oral intake of the toxins. Indeed, if okadaic acid and its derivatives are present, the following steps separate the injection of the toxin into the mice from the death of the animals: i) peritoneal shock, which enhances peritoneal resorption and modify the water and ione balance; ii) resorption of the injected toxin in the bold stream; iii) direct transport to the liver; iv) hydrolysis of acyl esters of the toxins (if present) with release of the active toxin; v) hepatotoxic effect; vi) severe metabolic disorders and death. ” If the amount of toxin(s) is low, the liver remains active and can secrete, trough the bile flow, part of the toxin in the bowel, where it may produce diarrhoea. The role of other non-diarrheogenic toxins (pectenotoxins and yessotoxins) is still unclear.

The long chain of events, that binds the presence of the toxins in the extract with the death of the mouse, results in a high variability of the biological response to the same amount of toxin. Consequently, the survival time of the mice represents only an approximate evaluation of the toxin content of the samples under examination. In other words, the Mouse Bioassay seems to respond to the question “yes or not” rather than to the question “how much”.

The test is strongly influenced by many factors, the most important of them is the general status of animals. The injection of a large volume of extract (5 % of the whole body weight) containing many foreign substances (the extractives of digestive grands corresponding to 25% of the animal weight) is very stressing. Therefore, if the animals are not in optimal condition, they will show survival times shorter than those espected for the actual toxin content and “false positive” responses are obtained. As a consequence, first quality animals have to be used and before the use they have to be allowed to recover from the eventual “traveling stress”. Also the weight of the used mice is critical: mice around the required weight (20 g) are in a fast growing phase and their weight may change by more than 1 g in a day. For this reason, the animals should be weighted just before use.

Many further experimental parameters can influence the results. of the Mouse Bioassay for DSP such as the history of samples before extraction, the extraction procedure, the purity grade of the used solvents and chemicals and so on. These parameters are easily standardized for a single laboratory, but differences in their standardization may lead to conflicting results between different laboratories that analyze the same sample.

TOXICOLOGICAL AND CHEMICAL ASPECTS OF PARALYTIC SHELLFISH POISONING (Dr. Helle Ravn)

Among all the seafood poisons PSP poses the most serious threat to public health, and economic damage caused by accumulation of the toxins in shellfish is immeasurable. More than 20 analogues of saxitoxin occur naturally. The chemistry of these analogues was describes in details.

Standards and reference materials are needed to perform reliable monitoring of seafood containing phytoplankton toxins, to develop and use chemical methods for quantitative determination and to perform toxicological tests.

One of the largest problems is to obtain pure standards or reference materials. Tables were presented containing information on where to obtain some of the standards and reference materials.

The most common PSP producing dinoflagellates belongs to the Alexandrium genus. Shellfish filtering these algae accumulate the toxins and the degree of accumulation depends on the species. PSP toxins have also been identified from a red alga called Jania sp. and bacteria as Moraxella sp. and Bacillus sp. The biology, origin, occurence, and distribution of PSP producing phytoplankton was described.

The epidemiology, absorption, mechanism of action, distribution, biotransformation and excretion of the PSP was presented. The symptoms observed by intoxication of PSP develop usually within 30 minutes from consumption of toxin or contaminated seafood with PSP toxins. The PSP toxins have a very specific action on the myelinated nervous tissue and skeletal muscle tissue in vertebrates. Both motor and sensory neurones are influenced. Paralysis in upper and lower limb may follow, manifesting itself in ataxia, loose of motor co-ordination, constrictive sensation in the throat, and incoherent speech. In case of high amounts of toxins, the paralysis can extend to the respiratory system, leading to respiratory arrest followed by death.

Concerning the treatment of PSP intoxication, no specific antidotes or drugs are available for direct cure of the intoxication. Artificial respiration is the only measure that can be taken.

The different chemical and biological quantitative and qualitative methods for analysis of PSP were described in details.

A copy of the lecture can be distributed by contacting: Dr. Helle Ravn, The IOC Harmful Algal Bloom Programme Office, Intergovernmental Oceanographic Commission (IOC) of UNESCO, 1, rue Miollis, 75732 Paris Cedex 15, Fax. :(33.1) 405693 16 e-mail: hab.ioc@unesco.org

THE AOAC MOUSE BIOASSAY FOR PSP: ANALYTICAL PROCEDURE AND FACTORS THAT CAN INFLUENCE THE RESULTS (Dr. Ana Martinez Fernandez)

The AOAC PSP Mouse Bioassay is the most used method for PSP determination with regulatory purposes. It was first applied by Sommer and Meyer (1937) and further standardized by the Association of Official Analytical Chemistry (AOAC). The assay consists of the following: 1 ml of the 0.1 N HC1 shellfish exctract obtained after 5' boiling is injected intraperitoneally in three standardized mice. The time between the injection and the mice death is used for the calculation of the PSP toxicity in mouse units. Mouse units are converted in mg Saxitoxin equivalents from dosis response curves obtained from a standard solution of saxitoxin. The tolerance level agreed by most countries is 80 mg of saxitoxin equiv. per 100 g of tissue.

Although this method has been validated and standardized, in practice there are some facts that can influence the variability within and among laboratories and the reliance of the assay.

Features of the mice such as general status, weight, sex and strain affect the results.

Overfeeding of the mice must be avoided. They may store between one and two grams of food in the digestive tract and the toxic response might not correspond to the weight measured. Animals weights from 17 to 19 g and from 21 to 23 g may be only used in the absence of animals in most desirable weight range. The use of mice weighing more than 23 g must be avoided.

The sensitivity of the assay very much depends on the mice strain and for this reason a periodical calibration of the mice used should be made.

An important parameter influencing the result is the pH during the extraction. In the AOAC procedure it is established a pH range between 2 and 4. The PSP procedure is initially designed to quantify only STX. Up to date about 24 different PSP analogues have been identified having different toxicity and stability. Among them, sulfocarbamoyl toxins are the less toxic. However, they are unstable and, under heating and strong acidic conditions (pH above 2), yield the most toxic carbamoyl toxin. The degree of conversion is dependent on the pH (Nagashima et al., 1991). As a result, a decrease in the pH range established in the AOAC procedure is highly advisable in order to reduce variability and improve reproducibility within a single laboratory and among different laboratories. At pH between 3-4, all the PSP components can be preserved. At a lower pH, changes in the toxin profile leading to higher toxicity might take place. The crucial issue is what is to be measured: potential maximum toxicity or real toxicity. The precautionary principle seems to defend the former, despite the possible over estimation of human health risk.

High amounts of salts (mainly sodium salt) dissolved in the extracts can decrease the PSP toxicity. The AOAC method recommends the addition of sodium hydroxide to rise the pH if necessary. This addition should be made carefully in order to avoid changes in the real toxicity of the samples. Moreover, if the addition is not carried out by continuously stirring, the NaOH might destroy the poison due to local alkalynization. At alkalyne pH, the atmospheric oxygen can oxydize the toxins yielding non-toxic substances.

It has been shown that high concentration of certain metals can be a source of interferences. Zn concentrations above 900 ppm, that might be present in some PSP-free bivalves produce death of mice upon intraperitoneal injection of extracts prepared according the AOAC PSP method (Mc Culloch et al., 1988; Cacho et al., 1993). Although symptoms are different from those characteristic of PSP they might lead to false interpretation depending on the experience of the assayist.

The AOAC PSP procedure can also detect domoic acid in concentration above 40 ppm and it was used when ASP was first identified in Canada in 1987 in shellfish extracts from eastern Prince Edward Island. The typical sign of the presence of domoic acid is a unique scratching syndrome of the shoulders by the hind leg followed by convulsions. The time of observation must be up to four hours. Deaths associated with mussels containing domoic acid were never observed after 135 minutes.

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THE USE OF RECEPTOR ASSAY FOR DETECTION OF PHYCOTOXINS: THEORY, PRACTICE AND APPLICATION (Prof. John S. Ramsdell)

Optimal utilization of marine resources requires reliable and practical methods to assure the safety of seafood before it reaches the marketplace.

The NOAA Southeast Fisheries Science Center Charleston Laboratory has worked to develop a reliable high capacity assay for shellfish toxins that is cost effective and utilizes no vertebrate animals. The use of receptors in place of antibodies is a reliable method for the detection of toxins in seafood because receptors recognize different toxins congeners in a manner quantitatively proportional to their toxicity. This minimizes false negatives and also provides a sum total toxicity value analogous to that of the mouse bioassay. The voltage dependent sodium channel is used for the detection of PSP, NSP and CFP toxins and the glutamate receptor (GluR6 subtype) is used for the detection of ASP toxins. Assays are based upon competition between toxin and radioligand for receptor sites and are modified for high capacity format using microplate scintillation technology to quantify multiple samples in a 96-place 4 x 5“ filter mat card. The ASP and PSP receptor assay have sensitivities of >1 ng and have been utilized to quantify toxin in algae, shellfish, crab viscera and the blood and urine of laboratory animals and humans.

DETERMINATION OF PSP TOXINS USING AN ELISA ASSAY (Dr. Alan D. Cerebella)

The introduction of immunological techniques to monitoring programs for the presence of phycotoxins in seafood and phytoplankton is an important advance in public health protection and may have considerable beneficial effect on acquaculture development. Derivatives of saxitoxin (STX), potent neurotoxins produced by toxigenic phytoplankton, accumulate in suspension-feeding shellfish resulting in an intoxication syndrome known as paralytic shellfish poisoning (PSP) in human consumers of contaminated shellfish.

A competitive inhibition enzyme-linked immunosorbent assay (ELISA) was developed with high specificity for STX, and polyclonal antibody also partially cross-reacts with some major gonyautoxin (GTX) analogues found in toxic dinoflagellates and in contaminated shellfish. The specificity and cross-reactivity of the anti-STX antibody was evaluated against purified STX derivatives, including NEO-STX, GTX2, GTX3, and a mixture of low potency N-21 -sulfocarbamoyl (C-) toxins. The STX-antibody exhibited a high affinity for STX, while cross-reacting to various degrees with the other derivatives tested. No reactivity was evident with the sulfocarbamoyl derivatives, nor against the tetrapurine tetrodotoxin. Other phycotoxins including domoic acid and okadaic and, Staphylococcus eterotoxin B also failed to cross-react.

This technique was incorporated into a rapid diagnostic kit (“Saxitoxin Test”; Institut Armand-Frappier), and compared with two standard methods for PSP toxins quantitation in shellfish tissues, the AOAC mouse bioassay and the fluorescence HPLC method, in a double blind experiment among four independent laboratories.

The ELISA test has a high potential sensitivity (1 pg saxitoxin equivalent [STX eq] per assay), although it may be configured to yield optimum detection in the range 1 to 80 µg STX eq/100 g shellfish tissue - the range of greatest concern for regulatory purposes. The results of the PSP assays by the ELISA kit and the HPLC and mouse bioassay methods were highly correlated; as expected, the ELISA test yielded most accurate values when the relative concentration of STX in the samples was highest. The ELISA test was also modified for the assay of PSP toxins in reference cultures of gonyaulacoid dinoflagellates and for natural phytoplankton blooms dominated by Alexandrium spp. from eastern Canada.

While the ELISA assay requires further refinement and this kit is no longer in commercial production, the relatively broad antigen specificity of the STX-antibody incorporated into the rapid test kit demonstrates its usfulness as a rapid diagnostic screening technique for PSP toxins in shellfish and toxic phytoplankton. The implications of STX-antibody specificity with respect to accurate diagnostics of samples containing high proportions of sulfated carbamate and N-sulfocarbamoyl toxins had been discussed. Quantitative and qualitative data on the efficacity, specificity, and sensitivity of alternative immunodiagnostic assays for PSP toxins currently under development were also addressed.

ANALYTICAL METHODS FOR DETERMINATION OF ASP TOXIS (Dr. Marisa Fernandez)

Domoic acid is a potent neurotoxic aminoacid responsible of amnesic shellfish poisoning. Although this compound is known since along time as a marine natural product, it was first associated with human neurotoxicity in 1987, when 153 people suffered an acute intoxication following the consumption of domoic acid contaminated blue mussel from Prince Edward Island in Canada. The planktonic diatom species Nitzschia pungens f. multiseries was identified as the source of the toxin in the mussels. A number of closely related compounds have been later detected in toxic mussels and in laboratory cultures of N.pungens. Some of them are isomers of DA and are also neurotoxic, although of lower potency. This family of compounds are currently referred to as ASP toxins. The ubiquitous and worldwide occurrence of potentially toxic Nitzschia spp. makes it necessary their inclusion in the monitoring programmes.

Domoic acid was first detected by using the Association of Official Analytical Chemists (AOAC) Mouse Bioassay for paralytic shellfish poisoning (PSP). It consists of an intraperitoneal injection into each of three standardized mice of the acidic aqueous extract obtained after boiling five minutes the contaminated mussel tissue with an equal volume of 0.1 N HC1. After the injection, typical symptoms of ASP as a scratching syndrome of the shoulders by the hind leg followed by convulsions are observed. The time of observation is extended up to four hours. 150 ppm of DA are required for repeatable time-to-death values. Deaths associated with contaminated mussels containing DA were never observed after 135 minutes.

During the AOAC extraction, a partial decomposition of the ASP toxins takes place, ranging a recovery yield between 75 and 85 %. Additional reported drawbacks of the AOAC procedure for ASP determination such as difficult extraction of the toxins with certain matrixes and, above all, low sensitivity of the assay (about 40 ppm that is above the tolerance level of 20 ppm established in Canada and subsequently adopted in other countries) makes it not suitable for regulatory purposes.

DA has a natural chromofore, a conjugated diene system that absorbes strongly at 242 nm, allowing its UV detection. Consequently, the AOAC Mouse Bioassy has been superseeded by isocratic or gradient HPLC methods using diode-array/UV or fluorimetric detection, the choice of the method mainly depending on the sensitivity required. Lawrence's method (1991) was adopted by the AOAC for official first action and uses the AOAC PSP Mouse Bioassay extraction procedure and reversed phase isocratic chromatography with UV detection at 242 nm. Although the extraction has the advantage of being suitable for both PSP and ASP determination, it must be born in mind that DA is not completely recovered by this procedure. The sensitivity is enough to detect DA in shellfish down to less than 1 ppm.

A rapid extraction with 50 % aqueous methanol yielding higher recoveries of DA (about 93%) and a selective clean-up and preconcentration with strong anion exchange solid phase extraction, that removes the tryptophan interference, has been proposed by Quilliam et al. (1995). The determination is performed by Liquid Chromatography with UV detection and is suitable for a wide range of marine products with a detection limit of 20-30 rig/g.

With the aim of improving the sensitivity, and detecting DA at trace level, a reversed phase gradient HPLC method with fluorimetric detection after derivatization with fluorenylmethoxicarbonyl chloride (FMOC) was developed by Pocklington et al. (1990). The method has been proven very useful for seawater and phytoplankton culture studies in field and laboratory, where a higher sensitivity is required.

A high throughput receptor assay for DA that makes use of the competition between [3H] kainic acid and DA for binding the kainate glutamate receptor in frog brain synaptosomes has been developed by Van Dolah et al. (1995). A good correlation between this assay and the HPLC analysis of DA in several marine matrixes was found.

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SAMPLING STRATEGIES OF ALGAE AND MUSSELS FOR MONITORING PURPOSES
(Dr. Laura Sidari)

The primary purpose of a monitoring programme for toxic events is public health protection. The secondary purpose is to ensure the quality of seafood products and to prevent the economical losses of shellfish farmers. A good monitoring programme must give reliable results in a short time. The most important things to take in account are: the choice of the sampling stations, the frequency of sampling, the collection of samples (potentially toxic microalgae considering both planktonic and benthic species; cysts in the sediment; filter feeding shellfish). The fixing and preserving agents should be chosen with regard to the aim of investigation.

The monitoring of potentially toxic microalgae by itself does not provide sufficient protection for public health, but can be used: 1) to provide useful early warnings for finfish and shellfish enterprises; 2) to establish a data base of toxic bloom events and associated ecological factors, for aquiculture site selection, risk assessment and trend analysis; 3) to determine the population dynamics and spatio-temporal distribution of toxic species (Cerebella and Todd, 1993).

The different ecology of potentially toxic species must be taken in account for monitoring purposes. The standard quantitative analysis of phytoplankton generally requires too much time to be used for the fast detection of potentially toxic species in seawater samples. On the other hand, a fast detection can be obtained using samples collected by vertical net howls for "semiquantitative" analysis (Sidari et al., 1995). Benthic potentially toxic species can be collected in association with macroalge and sediment samples Resting cysts of potentially toxic species are regarded as "seed population" for next blooms. Therefore, it is important to know which cysts are present in the studied area (qualitative analysis) and their abundance (quantitative analysis). From the analysis of the entire cores of sediment it is possible to know also the "history" of blooms in the area.

Normally, in monitoring programmes for PSP, DSP, NSP and ASP, only filter-feeding molluscs are considered. However, it is very important to include higher order consumers such as carnivorous, gastropod and crustaceans, in routine monitoring programs, especially in regions where non traditional species are being harvested (Shumway, 1995). In some cases also the analysis of phytoplankton in the stomach content of shellfish can be useful.

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ANNEX VI

PROCEDURES AND EXPERIMENTAL RESULTS

DETERMINATION OF DSP

During the practical part, three different methods to detect DSP toxins were carried out by each participant. Two mussel samples, at different degree of contamination (samples A and B), were analyzed by the Mouse Bioassay, by an ELISA assay, and by a protein phosphatase inhibition assay. To allow a comparison between the results obtained by the three methods used, each participant examined the same samples. Furthermore, the same samples were analyzed by all the participants and the results obtained by everyone were also compared to evaluate the personal intervariability.

Sample A (kindly supplied by the European Communities Reference Laboratory of Vigo, Spain) had a DSP toxin concentration of about 3.35 µg/g digestive glands, determined by HPLC, mainly represented by okadaic acid (about 3.10 µg/g) and by acylderivatives of okadaic acid (0.25 µg/g). The HPLC determination was carried out two months before the course. Sample B was obtained diluting 1:3 the Sample A with non contaminated digestive glands and contained theoretically 0.82 µg/g okadaic acid.

A demonstration of HPLC analysis for okadaic acid was carried out too, using samples different from A and B.

DSP Determination by the Mouse Bioassay (Dr. S. Sosa)

The participants were divided in six groups of two or three participants and each participant of the same group extracted the same sample. Participants 1, 3, 5, 7, 9 and 12 extracted sample A, whereas participants 2, 4, 6, 8, 10, 11 extracted sample B. The mouse bioassay was carried out according to the Italian law (G. U., 1990). Homogenized mussel digestive glands (20 g) were extracted three times with acetone (100, 50 and 50 ml) and the extracts filtered. The filtrates were pooled and the solvent was evaporated by rotavapor at 30-40° C. The residue, suspended in NaCl saturated water, was extracted three times with 50 ml ethyl ether each time. The organic layers were pooled, washed three times and then evaporated to dryness. The residue was suspended in 4 ml of 1 % Tween 60 aqueous solution. One ml of the suspension was injected intraperitoneally in three CD-1 female mice (Charles River, Calco, Italy), weighing 18-20 g. The animals were observed for 24 hours after the treatment and their survival times recorded. From the data obtained by each participant, the mean and median survival times for the two tested samples were calculated.

The obtained results are reported in Table I. Homogeneous data were recorded using the highly contaminated sample with mean and median survival times of 0.6 and 0.7 hours, respectively. In accordance to previous observations that a decrease of the sample toxicity increases the variation in survival time (Stabell et al., 1992), more variable results were obtained testing the sample B, with survival times ranging from 0.9 to 20 hours. Anyway, both the mean and the median survival times (4.5 and 2.6 hours) were below 5 hours and therefore, according to the Italian law, both the samples were positive.

Tab. I: Mouse bioassay for DSP toxins.

SAMPLE A	Survival times (hours)				
Particip.	Mouse 1	Mouse 2	Mouse 3	Mean	Median
1	0,7	0,5	0,6	0,6	0,6
3	0,8	0,7	0,7	0,7	0,7
5	0,6	0,6	0,6	0,6	0,6
7	0,6	0,7	0,6	0,6	0,6
9	0,7	0,5	0,6	0,6	0,6
12	24	0,8	0,6	0,7	0,8
Mean				0,6	0,7
SAMPLE B	Survival times (hours)				
Particip.	Mouse 1	Mouse 2	Mouse 3	Mean	Median
2	2,0	2,5	2,6	2,4	2,5
4	1,7	1,3	6,1	3,0	1,7
6	2,0	1,5	1,9	1,8	1,9
8	5,8	5,5	20,0	10,4	5,8
10	2,3	1,7	20,0	8,0	2,3
11	0,9	1,5	2,3	1,6	1,5
Mean				4,5	2,6

References:

Gazzetta Ufficiale della Repubblica Italiana n. 211. 10 settembre 1990. Decreto Ministerial n. 256 e Decreto Ministerial n. 257, 1 agosto 1990.

Stabell O. B., Steffenak I., Aune T. “An evaluation of the mouse bioassay applied to extracts of Diarrhetic Shellfish Toxins”. Fd. Chem. Toxicol. 30: 139-144 (1992).

DSP Determination by ELISA (Dr. A. Tubaro and Dr. L. Cerni)

After a detailed introduction concerning the possibilities to use an ELISA assay in a qualitative and semi-quantitative way for the assessment of DSP toxins, the advantages and the disadvantages of the method were discussed. The assay was carried out, as described by Della Loggia et al. (1993), with a commercially available competitive enzyme immunoassay kit (DSP-Check, Panapharm, Japan).

The kit takes advantage of a peroxidase-conjugated monoclinal antibody, sensitive both to okadaic acid and dinophysistoxin-l (Usagawa et al., 1989). A constant quantity of okadaic acid is fixed on immunoplate wells, provided with the kit. When the sample under testing and the monoclinal antibody solutions are added into the wells, the antibody binds to the fixed okadaic acid in disproportion to the concentration of free okadaic acid from the sample. After washing the plate, the amount of fixed peroxidase-conjugated antibody is determined in terms of enzyme activity, which can be measured photometrically after a calorimetric substrate reaction. The colour development is inversely proportional to the toxin concentration (Uda et al., 1989).

Each of the participants prepared the extracts from samples A and B. By Ultra-Turrax homogenization at 5,000 rpm for 90 see, 0.5 g digestive glands of each of the two samples were extracted in 2.5 ml of 90 % aqueous methanol. The homogenate was centrifuged for 5 min at 15,900 g, the supernatant was diluted with twice-distilled water (1:1) and then centrifuged again for 5 min at 15,900 g. The obtained supernatant was used for the ELISA assay, that was carried out according to the instructions of the producer. Each sample was tested in duplicate.

In each well of the immunoplate, 50 µl okadaic acid standard solutions (100, 55, 32.5 and 10 rig/ml) or sample solutions were added. Immediately, 50 µl of the antibody solution was added, allowing to stand for 10 minutes while gently shaking. The well content was then poured out and the wells washed four times with the washing solution. To reveal the amount of peroxidase labelled antibody, 50 µl of chromogen-substrate solution were added into each well. The enzymatic reaction was allowed to go on for 6 minutes in the dark, then it was stopped by the addition of 50 µl of stopping reagent. The plate was shaked and, after 3 minutes, the absorbance was read by a multiplate autoreader (EL 311s - Biotek Instruments, USA) at 492 nm.

The results obtained by each participant are reported in Table II. The toxin content of sample A (about 3.35 µg/g digestive glands) was out of the working range of the kit (O. 1 to 1.0 µg of OA and/or DTX-1/g DG) and the obtained results were very variable. More homogeneous results were obtained with sample B, although the amount of toxins was underestimated also in this case. This could be due to a critical step in the procedure represented by the stability of the diluted methanolic extract of the mussel digestive glands. Previous observations revealed that the time passing between preparation of the 45 % methanolic extract and the ELISA assay is critical for possible toxin losses (Tubaro et al., 1992).

Tab. II: ELISA assay for DSP toxins.

	DSP toxin concentration µg/g)	
Particip.	Sample A	Sample B
1	1,20	0,50
2	0,68	0,36
3	0,84	0,45
4	0,75	0,45
5	0,56	0,38
6	0,64	0,46
7	1,00	0,60
8	0,62	0,46
9	1,00	0,70
10	0,27*	0,15
11	0,60	0,31
12	0,68	0,46
13	0,70	
Mean	0,77	0,47
SD	0,20	0,11

* Not considered value: operative mistakes.

References:

Della Loggia R., Cabrini M., Del Negro P., Honsell G.. Tubaro A. “Relationship between Dinophysis spp. in seawater and DSP toxins in the Northern Adriatic Sea”. Developments in Marine Biology 3: 483-488 (1993).

Tubaro A., Sosa S., Bruno M., Gucci P, M. B., Volterra L., Della Loggia R. “Diarrhoeic shellfish toxins in Adriatic Sea mussels evaluated by an ELISA method”. Toxicon 30: 673-676 (1992).

Uda T., Itoh Y., Nishimura M., Usagawa T., Murata M., Yasumoto T. “Enzyme immunoassay using monoclinal antibody specific for diarrhetic shellfish poisons”. In “Mycotoxins and Phycotoxins‘88”, Natori S., Hashimoto K., Ueno Y. Eds., Elsevier Science, Amsterdam 1989, pp. 335-342.

Usagawa T., Nishimura M., Itoh Y., Uda T., Yasumoto T. “Preparation of monoclinal antibodies against okadaic acid prepared from the sponge Halichondria okadaii ”. Toxicon 27: 1323-1330 (1989).

DSP Determination by a protein phosphatase 2a inhibition assay (Dr. C. Florio and Dr. E. Luxich):

This new method takes advantage upon the mechanism of action of okadaic acid, a selective inhibitor of protein phosphatase type-2A (PP2A), and upon the ability of PP2A to dephosphorylate, a colorless substrate (p-nitrophenyl phosphate, p-NPP) to a yellow product (p-nitrophenol, p-NP). In this way, being okadaic acid an inhibitor of PP2A, it can inhibit the PP2A-induced transformation of p-NPP into the coloured p-NP. Thus the reduction of the colour development can be spectrophotometrically measured.

All the participants prepared the extracts of samples A and B, according to the following procedure. One gram of DG was homogenized by Ultra-Turrax in 4 ml of 80 % methanol for 2 min at 5,000 rpm. The suspension was centrifuged for 5 min at 1,600 g and 3 ml of the supernatant were washed twice with 3 ml hexane. The lower phase was separated, diluted with water to about 50 % methanol, and subsequently extracted twice with the same volume of ethyl acetate. The organic phases were pooled, dried on sodium sulphate and, after filtration, the solvent was removed by rotovapor. Immediately before testing, the residue was dissolved in the appropriate amount of ethanol to obtain an extract concentration corresponding to 1 g DG/ml. Before to carry out the assay, this solution was further diluted in the incubation buffer.

The protein phosphatase inhibition assay was carried out according to Tubaro et al. (1995), using a commercially available protein phosphatase type-2A (Upstate Biotechnology Incorporated, New York, USA). The assay was carried out in 96 multiwell plates, each well containing 100 µl buffer (40 mM Tris/HCl pH 8.4, containing 34 mM $MgCl_2$, 4 mM EDTA and 4 mM DL-dithiothreitol), 50 µl substrate (28.2 mM final concentration) and 50 µl okadaic acid standard (ranging from 0.06 to 1.5 ng/ml) or 50 µl mussel extract. The reaction was started by the addition of 50 µl of the enzyme (0.05 U/ml final concentration) and carried out for 1 hour at room temperature, The hydrolysis of the substrate was recorded on a Microplate Autoreader (Biotek Instruments, USA) at 405 nm.

In Table III the obtained results are reported. A mean concentration of 2.43 µg okadaic acid per gram of digestive glands was revealed for sample A, whereas a mean concentration of 0.61 µg/g was obtained for sample B.

Tab. III: PP2A-inhibition assay

Particip	DSP toxin concentration (µg/g) Sample A	Sample B
1	1,16*	0,63
2	2,78	0,63
3	2,10	0,46
4	2,28	0,48
5	2,90	0,98
6	2,.61	0,88
7	4,10*	0,60
8	3,20	0,63
9	1,50	0,47
10	2,60	0,44
11	2,30	0,52
12	2,10	0,52
13		0,70
Mean	2,43	0,61
SD	0,49	0,16

* Not considered values: operative mistakes.

References:

Tubaro A., Florio C., Luxich E., Sosa S., Della Loggia R., Yasumoto T. “A fast and highly sensitive assay to detect okadaic acid in mussels”. Abstract - Seventh International Conference on Toxic Phytoplankton - Sendai, July 12-16, 1995.

DSP Determination by HPLC (Dr. C. Casadei):

After a detailed introduction on the various extraction, clean-up and derivatization procedures for okadaic acid detection by HPLC, reported in literature, the mussel extraction procedure was described (Lee et al., 1987). One gram DG has to be homogenized with 4 ml 80 % aqueous methanol and centrifuged at 1,600 g for 5 minutes. The supernatant (2.5 ml) must be washed twice with 2.5 ml petroleum ether to remove lipid components. After centrifugation at 1,600 g for 2 minutes and the subsequent removal of the ether layer, 1 ml of water and 4 ml of chloroform must be added. The mixture is shaken vigorously and centrifuged at 1,600 g for 5 minutes. The lower layer (chloroform phase) has to be separated whereas the upper layer (petroleum ether) is extracted again with chloroform. The chloroformic extracts are combined and made up to 10 ml. An aliquot of the chloroform extract (0.5 ml) is dried under nitrogen and added with 100 µl of 0.1 % 9-anthryl diazomethane solution for the toxin derivatization, that takes 1 hour in dark at 25°C. After evaporating the solvent, the residue is dissolved twice in 0.3 ml of hexane:dichloromethane (1: 1) and transferred on a sep-pack silica cartridge column (Waters, Co) for the clean-up procedure. The first rinsing is effected with 5 ml of the loading solution while the second one is carried out with 5 ml of dichloromethane. The elution of the derivatized toxin is carried out with 5 ml of dichloromethane: methanol (9: 1). The eluated is collected and the solvent evaporated in rotavapor at a temperature below 30” C. The residue is dissolved in 100 µl of methanol and 10 µl are used for HPLC injection.

Two mussel samples previously extracted and derivatized, were used for the HPLC analysis demonstration. A Perkin Elmer chromatography equipped with a luminescence spectrometer model LS 30 and a binary LC pump model 250 was used. The toxin analysis was carried out on a Supelcosil LC18 column (25 cm x 4.6 mm, 5 µm, Supelco) using acetonitrile:methanol :water (8: 1: 1) as the mobile phase and the flow rate at 1.1 ml/min. The excitation and emission wavelengths were set at 365 and 412 nm, respectively.

References:

Lee J, S., Yanagi T., Kenma R., Yasumoto T. "Fluorometric determination of Diarrhetic Shellfish Toxins by high-performance liquid chromatography". Agric. Biol. Chem. 51: 877-881 (1987),

DETERMINATION OF PSP

Two different methods, the AOAC mouse bioassay and a radio-receptor binding assay, were used to carry out PSP toxin analysis on two mussel samples (sample 1 and 2), received from the European Community Reference Laboratory of Vigo. Each participant prepared one mussel extract that was subsequently analyzed by the two methods. Other two mussel samples (samples 3 and 4), algal extracts (samples 5 and 6), and a PSP contaminated human urine sample (sample 7) were tested at the radio-receptor binding assay too. Furthermore, a demonstration of an ELISA assay for PSP toxins was performed.

PSP determination by the AOAC mouse bioassay (Dr. A. ,Martinez):

After an introduction on the mouse bioassay of the AOAC, each participant prepared his own sample to be analyzed, as described by Hollinworth and Wekell (1990). Participants were divided in six groups of two or three and each participant of the same group extracted the same mussel sample: participants 1, 3, 5, 7, 9, and 11 extracted sample 1, while participants 2, 4, 6, 8, 10, 12, and 13 extracted sample 2. Due to the variability of the mice response, before to carry out the mouse bioassay, the standardization of mice was carried out in order to calculate the conversion factor (the amount of PSP toxins equivalent to 1 MU), that is necessary for the determination of PSP toxin concentration in the samples under testing.

Mice standardization:

The standardization was carried out on CD-1 female mice weighing 19-21 g (Charles River, Calco, Italy), using a PSP standard solution (saxitoxin dihydrochloride 100 µl/ml, Food and Drug Administration). Since a variability of mice response can occur also in the same laboratory if the bioassay is carried out for the first time, the official procedure recommends to perform the standardization repeating all the procedures in different days. For time reasons, the mice standardization was carried out in only one day.

A working PSP standard solution was prepared from the standard solution by diluting it to the concentration of 1 µg/ml with distilled water. Aliquots of 10 ml of the working standard solution were diluted with 10-15-20-25-30 ml of water and the pH of the diluted solutions was measured and adjusted to 3. One ml of the prepared solutions was injected into groups of 3 mice, intraperitoneally. The dilution which caused the animal's death in a median time of 5-7 minutes was chosen and it was tested again with variations of $\pm$ 1 ml of water. Groups of ten mice were injected with 1 ml of each of three dilutions prepared as above and the death time was observed. The dilutions that give median death times between 5 and 7 minutes were chosen and the median death time for each group of ten mice was converted into Mouse Units/ml, using Sommer's Table (see Recommended Procedures for the Examination of Sea Water

and Shellfish, 1970). The toxin concentration calculated for 1 ml was then divided by the MU/ml to obtain the conversion factor, that resulted to be 0.20 µg saxitoxin dihydrochloride/MU.

The official procedure recommends to repeat the test one or two days later, using the same dilutions described above ± 1 ml of water. Moreover, the whole test should be repeated starting with new dilutions obtained from the standard working solution (lµg/ml), prepared just before using. Groups of ten mice have to be inoculated with the prepared dilutions and the median death time has to be calculated for each group. The dilutions that give median death times between 5 and 7 minutes are chosen for the calculations. The median death time for each group often mice gives the MU/ml, that can be transformed into the conversion factors, as previously described. The mean correction factor is then calculated: this value is used as reference point to check the routine tests. When routine analyses are performed using the AOAC mouse bioassay, the CF has to be checked, injecting at least five mice with the working standard dilutions.

Extract preparation and mouse bioassay:

After the mice standardization, the mussel extracts from sample 1 and 2 were prepared. Homogenized mussels meat (100 g) were mixed with 100 ml 0.1 N HCl and the pH adjusted to 3. The mixture was boiled for 5 minutes and, after cooling at room temperature, the pH was determined again and adjusted to pH 3. After adjusting the volume to 200 ml with 0.003 N HCl, the extract was centrifuged at 1,600 g for 5 min.

One ml of the supernatant was injected intraperitoneally into each of three standardized mice weighing 19-21 g and the survival time was recorded. The obtained results are reported in Table IV. In the case of sample 1, the extracts had to be diluted because the obtained values' were out of the linear range of the method (death time between 5 and 7 minutes). Since there were not sufficient mice of the appropriate weight, the dilutions were made only for two extracts and the toxin concentration resulted to be quite the same in both extracts tested (678 and 671 µg saxitoxin dihydrochloride equivalents/100 g meat). Sample 2 gave more variable results, with toxin concentrations ranging from 125 to 377 µg saxitoxin dihydrochloride equivalents/100 g of meat. According to the Italian law, both the samples were positive for PSP toxins, since they exceeded the the tolerance level of 80 µg saxitoxin equivalents/100 g of meat.

Tab. IV: Mouse bioassay for PSP toxins

Particip.	Sample 1 µg STX/100 g	Particip.	Sample 2 µg STX/100 g
1	678	2	125
3	671	4	350
5	-	6	141
7	-	8	377
9	-	10	167
11	-	12	209
		13	136

References:

Hollinworth T., Wekell M. In "Official Methods of Analysis of the AOAC", Hellrich Ed., Arlington, Virginia 1990, pp. 881-882.

"Recommended Procedures for the Examination of Sea Water and Shellfish", 4th Edition, American Public Health Association, 1970.

PSP determination by a radio-receptor binding assay (Prof. J.S. Ramsdell)

A description and a practical demonstration of a competitive receptor binding assay for saxitoxin was carried out. The assay is based upon competition between [^{3}H]-saxitoxin and native toxin for receptor sites and is modified for high capacity format using microplate scintillation technology to quantify multiple samples in a 96-well filter plate with glass filter. Crude mouse brain synaptosomes were used as source of receptor, whereas the analyzed samples were four extracts prepared by the standard AOAC extraction protocol from the mussels (two extracts of the sample 1 and two of the sample 2). Furthermore, two extracts of contaminated mussels collected in Alaska (samples 3 and 4), two algal samples obtained from Alexandrium sp. (samples 5 and 6) and one human urine sample (sample 7) were tested too. The assay included an intra-assay control sample as well as serial dilutions of one of the mussel samples to verify the slope of the standard curve. Each sample was run in triplicate.

Into each well of a 96-well filter plate with glass filter (type C), radiolabelled saxitoxin ([^{3}H]-STX, 0.84 nM final concentration), standard saxitoxin (0.1-100 nM final concentration) or sample solution diluted between ten and thousand fold (34 µl) and tissue preparation (134 µl preparation containing about 0.5 mg/ml protein) were added. The plate was incubated for 1 hour at 4° C and then filtered on a Millipore-Multiscreen Vacuum Manifold. A multichannel pipette was used to rinse the plate once with 200 µl of ice cold HEPES buffer (75 mM pH 7.5, containing NaCl 140 nM) per well. The filters on the bottom of each well were punched into omni vials pre-filled with liquid scintillant and each vial vortexed. After allowing vials to sit overnight, the radioactivity was measured in a beta counter. The IC50 for the standard curve was 1.0 nM. The IC10 and IC90 were 0.1 an 10 nM, respectively. The serial dilution of the mussel extract gave values parallel with the standard curve. The results obtained are reported in Table V and reported as the mean of three replicates. The toxin concentration found in the two extracts prepared from sample 1 were 506 µg/100 g and 850 µg/g, whereas the toxin concentration in the two extracts prepared from sample 2 gave a toxicity of 338 µg/100 g and 66 µg/100 g. The toxin concentration from two mussel samples from Alaska (samples 3 and 4) was 849 and 1343 µg/100g. The toxin concentration in the culture medium of two Alexandrium samples were 15,100 and 18,000 nM and the toxin concentration in the human urine was 47.4 nM.

Tab. V: Saxitoxin concentration evaluated by the receptor binding assay

Sample	cpm	Extract solutions cone.		Original sample cone.	
		nM	pg/1	µg/100 g	nM
1	1660	1.06	2.53	506	-
1	1224	1.78	4.25	850	-
2	1882	0.71	1.69	338	-
2	2389	0.14	0.33	66	-
3	1048	2.37	5.66	849	-
4	719	3.75	8.95	1343	-
5	919	2.51	-		15,100
6	834	3.00	-		18,000
7	1889	0.79	-		47.4
I-AC*	1512	1.13	-		6.78

* Intra-assay control

PSP determination by ELISA (Dr. A.D. Cerebella):

A canadian dipstick shellfish ELISA test kit for determination of saxitoxin (Saxitoxine-Test*, Institut Armand-Frappier, Laval, Québec) was introduced and demonstrated. The method is based on an inhibition-enzyme immunoassay technique, that requires a saxitoxin-coated plastic stick and a tube containing a fixed amount of anti-saxitoxin antibody capable of binding the saxitoxin present in the sample under analysis. The assay starts by plunging the saxitoxin-coated stick into a tube containing the sample under analysis and the anti-saxitoxin antibody for 5 minutes. A complex saxitoxin-antibody-saxitoxin will be formed. If the sample contains saxitoxin, it will bind to the antibody in the tube and this will inhibit further reaction with the saxitoxin on the stick. To reveal the complex formation, the stick is washed and then dipped into a tube containing 0.3 ml protein A-peroxidase conjugate for 5 minutes. The conjugate bound to the antibody is revealed by washing the stick and transferring it into a couvette containing 1 ml of chromogen-substrate solution for 5 minutes. The stick is then removed and the reaction is stopped by adding 0.5 ml of the stopping solution. The absorbance is read on a spectrophotometer at 450 nm.

ANNEX VII

FINAL EVALUATION OF THE EXPERIMENTAL RESULTS

During the practical part, DSP and PSP contaminated mussel samples were extracted and tested by all the participants, using different methods for each sample (see Annex VI). In this way, the obtained results allowed a comparative analysis based on the used methods. Furthermore, the intra- and inter-assay variability of untrained operators could be evaluated.

EVALUATION OF DSP TOXICOLOGICAL RISK

Two mussel samples, at different degree of contamination (Samples A and B), were analyzed by the Mouse Bioassay, by an ELISA assay and by a protein phosphatase inhibition assay. Sample A was kindly supplied by the European Community Reference Laboratory of Vigo, Spain. It was constituted by homogenized and frozen digestive glands of mussels, containing about 3.35 µg/g of DSP toxins, mainly represented by okadaic acid (about 3.10 µg/g) and by acylderivatives of okadaic acid (about 0.25 µg/g). The declared toxin content of the sample was determined by HPLC two months before the Course. Sample B was obtained diluting 1:3 the Sample A with non contaminated mussel tissue (final concentration: 0.82 µg/g of DSP toxins).

Table I summarize the results obtained by the participants with Sample A, using the three different methods. For time-table reasons, the Mouse bioassay on Sample A was carried out by six of the participants, whereas the remaining seven participants analyzed Sample B. The results obtained for Sample B are reported in Table II.

Tab. I: Sample A - comparison of results obtained by three methods of analysis.

Participant	Mouse bioassay - Survival times (hours)					ELISA	PP2A
	Mouse 1	Mouse 2	Mouse 3	Mean	Median	µg/g	µg/g
1	0,7	0,5	0,6	0,6	0,6	1,20	1,16*
2						0,68	2,78
3	0,8	0,7	0,7	0,7	0,7	0,84	2,10
4						0,75	2,28
5	0,6	0,6	0,6	0,6	0,6	0,56	2,90
6						0,64	2,61
7	0,6	0,7	0,6	0,6	0,6	1,00	4,10*
8						0,62	3,20
9	0,7	0,5	0,6	0,6	0,6	1,00	1,50
10						0,27’	2,60
11						0,60	2,30
12	>24	0,8	0,6	0,8	0,8	0,68	2,10
13						0,70	
Overall mean				0,6	0,7	0,77	2,43
SD				0,1	0,1	0,20	0,49
CV (%)				16,7		26,0	20,2

* Values not included in the mean calculation because of procedure errors.

* Values not included in the mean calculation because of procedure errors.

Table II: Sample B - comparison of results obtained by three methods of analysis.

Participant	Mouse bioassay - Survival times (hours)					ELISA	PP2A
	Mouse 1	Mouse 2	Mouse 3	Mean	Median	µg/g	µg/g
1						0,50	0,63
2	2,0	2,5	2,6	2,4	2,5	0,38	0,63
3						0,45	0,46
4	1,7	1,3	6,1	3,0	1,7	0,45	0,48
5						0,38	0,98
6	2,0	1,5	1,9	1,8	1,9	0,46	0,88
7						0,60	0,60
8	5,8	5,5	20,0	10,4	5,8	0,46	0,63
9						0,70	0,47
10	2,3	1,7	20,0	8,0	2,3	0,15*	0,44
11	0,9	1,5	2,3	1,6	1,5	0,31	0,52
12						0,46	0,52
13							0,70
Overall mean				4,5	2,6	0,47	0,61
SD				3,7	1,6	0,11	0,16
CV (%)				82,2		23,4	26,2

*Value not included in the mean calculation because of procedure errors

According to the Italian law (G. U., 1990), both the samples were positive for DSP toxins since the mean survival times at the mouse bioassay were below the tolerance limit of 5 hours. Using this method, the most contaminated sample (sample A) gave more homogeneous results than the less contaminated sample (sample B). For a global evaluation of the mouse bioassay results, the overall mean and the median of the survival times for the two samples were calculated. These values were similar for the sample A ((),6 and 0.7 hours, respectively), Sample B gave more variable results and the difference between mean and median of the survival times was high (4.5 and 2.6 hours, respectively), This can be explained by the high variability of the mice response when low contaminated samples are tested.

The theoretical ratio between the toxin concentration of sample B and sample A was 0.25 (dilution 1:3). When the overall means of the survival times are considered, the ratio between sample A and sample B survival times is O. 13. On the contrary, if the overall medians are considered, the ratio value (0.27) is close to that expected (0.25). This kind of results suggests that the median of the survival times is a parameter for the evaluation of the toxin content better than the mean,

Concerning the ELISA assay, variable results were obtained testing Sample A, However. its toxin content was out of the working range of the kit (0.1-1.0 µg OA and/or DTX-1/g DG) and its concentration could be not properly estimated. A toxins concentration of 0.47 µg/g DG was measured in sample B. In this case. the ratio between the toxin concentrations in the two samples could not be calculated, in order to verify if it corresponded to the theoretical value of 0.25. The low concentration detected for sample B could be due to toxin losses (Tubaro et al., 1992) and to the presence of OA acyklerlvatlves not detectable by the ELISA system.

At the Protein Phosphatase 2A inhibition assay, an okadaic acid concentration of 2.43 µg/g DG was determined in sample A. while 0.61 µg/g were revealed in sample B: the ratio between sample B and sample A concentrations was 0,25, as expected. The PP2A activity inhibition by OA acylderivatives, present in the samples A and B, is lower than that by OA. This can parrially account for the lower contamination determined

by the PP2A inhibitory assay. Anyway, since after the HPLC analysis the sample A was stored at -20°C for two months, a certain loss of toxins could be taken in account.

Considering the toxins concentration previously determined by HPLC analysis, the concentrations assessed by the two, biochemical methods were lower than those expected. However, a good correlation between the biological and the two biochemical analyses was found. In fact, short survival times of mice correspond to high toxin content (sample A), while longer survival times correspond to lower toxin content (sample B). Furthermore, sample B toxin concentration measured by the protein phosphatase 2A inhibition assay was somewhat higher than that obtained by the ELISA assay. Nevertheless, the difference between the results obtained by the two methods was not statistically significant.

A general consideration has to be finally underlined: the extraction procedures of mussel samples are quite different, depending on the used tests. Therefore, the OA yield can be different, according to the extraction procedure adopted. This variability could account for the different values recorded by the different methods.

The intra-assay variability for untrained operators, as the participants were, ranged from 17 to 26 % for sample A and from 23 to 82 % for sample B. In particular the highest variability observed at mouse bioassay (82 %) with the less contaminated sample B largely depends on the variability of mice response, as already underlined. The inter-assay variability for ELISA ranged from 23 to 26 %, while for the PP2A inhibition assay ranged from 20 to 26 70, independently from the-sample contamination.

Considering that all the participants carried out most of these assays for the first time, we can conclude that a good intra-assay correlation was reached. In addition, considering the results obtained using the different methods, we can conclude that, in general, a good correlation was obtained between the mouse bioassay, the HPLC and the PP2A inhibition assay results for the most contaminated sample (A), despite of the different extraction procedures used. Furthermore, the results obtained for sample B using the ELISA and PP2A inhibition assay are also in good agreement.

References:

Gazzetta Ufficiale della Repubblica Italiana n. 211. 10 settembre 1990. Decreto Ministeriale n. 256 e Decreto Ministeriale n. 257, 1 agosto 1990.

Tubaro A., Sosa S., Bruno M., Gucci P. M. B., Volterra L., Della Loggia R. “Diarrhoeic shellfish toxins in Adriatic Sea mussel evaluated by an ELISA method”. Toxicon 30: 673-676 (1992).

EVALUATION OF PSP TOXICOLOGICAL RISK

The assessment of the PSP toxicological risk was carried out using the AOAC Mouse Bioasssay and a receptor-binding assay (see Annex 6). Two mussel samples received from the European Community Reference Laboratory of Vigo (Samples 1 and 2: PSP toxin content not declared) were used and the results are reported in Table III.

For technical reason, Sample 1 was submitted at the mouse bioassay only by two participants, wich obtained very similar results. Sample 2 was tested by seven participants at the mouse bioassay, with not very homogeneous results (CV=46%). Only two analyses were carried out using the receptor assay, with variable results. Anyway, the means of the toxin content found by means of the two tests was similar both for Sample 1 and for Sample 2, but due to the low number of analyses carried out and to the variability of untrained people, it is not possible to set up any definitive conclusion.

Tab. III: Comparison of results obtained by two methods of analysis.

	sample 1 (µg STX/100g)			Sample 2 (µg STX/100g)	
Participant	Mouse bioassay	Receptor assay	Participant	Mouse bioassay	Receptor assay
1	678	506	2	125	338
3	671	850	4	350	66
5			6	141	
7			8	377	
9	.	.	10	167	
11			12	209	
			13	136	
Mean	675	678		215	202
SD				98	
CV (%)				46	

EVALUATION OF PSP TOXICOLOGICAL RISK

The assessment of the PSP toxicological risk was carried out using the AOAC Mouse Bioasssay and a receptor-binding assay (see Annex 6). Two mussel samples received from the European Community Reference Laboratory of Vigo (Samples 1 and 2: PSP toxin content not declared) were used and the results are reported in Table III.

For technical reason, Sample 1 was submitted at the mouse bioassay only by two participants, wich obtained very similar results. Sample 2 was tested by seven participants at the mouse bioassay, with not very homogeneous results (CV=46%). Only two analyses were carried out using the receptor assay, with variable results. Anyway, the means of the toxin content found by means of the two tests was similar both for Sample 1 and for Sample 2, but due to the low number of analyses carried out and to the variability of untrained people, it is not possible to set up any definitive conclusion.

Tab. III: Comparison of results obtained by two methods of analysis.

	Sample 1 (µg STX/100g)			Sample 2 (µg STX/100g)	
Participant	Mouse bioassay	Receptor assay	Participant	Mouse bioassay	Receptor assay
1	678	506	2	125	338
3	671	850	4	350	66
5			6	141	
7			8	377	
9			10	167	
11			12	209	
			13	136	
Mean	675	678		215	202
SD				98	
CV(%)				46	

ANNEX VIII

LIST OF ACRONYMS

ADAM	9-Anthryldiazomethane
AOAC	Association of Official Analytical Chemists
ASP	Amnesic Shellfish Poisoning
AST	Amnesic Shellfish Toxin
CF	Conversion Factor
CFP	Ciguatera Fish Poisoning
CV	Coefficient of Variation
DA	Domoic Acid
DG	Digestive Gland
DSP	Diarrhetic Shellfish Poisonig
DST	Diarrehetic Shellfish Toxin
DTX-1	Dinophysistoxin-1
DTX-2	Dinophysistoxin-2
EC	European Community
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FMOC	Fluorenylmethoxicarbonyl chloride
GTX 1-4	Gonyautoxins 1-4
HACCP	Hazard Analysis of Critical Control Points
HPLC	High Pressure Liquid Chromatography
IEO	Instituto Español de Oceanografía
IOC	Intergovernmental Oceanographic Commission
IPH	Institute of Public Health
MAP	Mediterranean Action Plan
MU	Mouse Unit
NSP	Neurotoxic Shellfish Poisonig
NST	Neurotoxic Shellfish Toxin
OA	Okadaic Acid
p-NPP	p-Nitrophenylphosphate
p-NP	p-Nitrophenol
PP2A	Protein Phosphatase 2A
PSP	Paralytic Shellfish Poisoning
PST	Paralytic Shellfish Toxins
SD	Standar Deviation
STX	Saxitoxin
UNEP	United Nations Environment Programme
WHO	World Health Organization