united nations
educational, scientific
and cultural
organization

international atomic
energy agency

the
abdus salam
international centre for theoretical physics

SMR 1550 - 11

WORKSHOP ON THE USE OF RECEPTOR BINDING ASSAY (RBA)

1 - 5 September 2003

Co-organized by the International Atomic Energy Agency (I.A.E.A.)

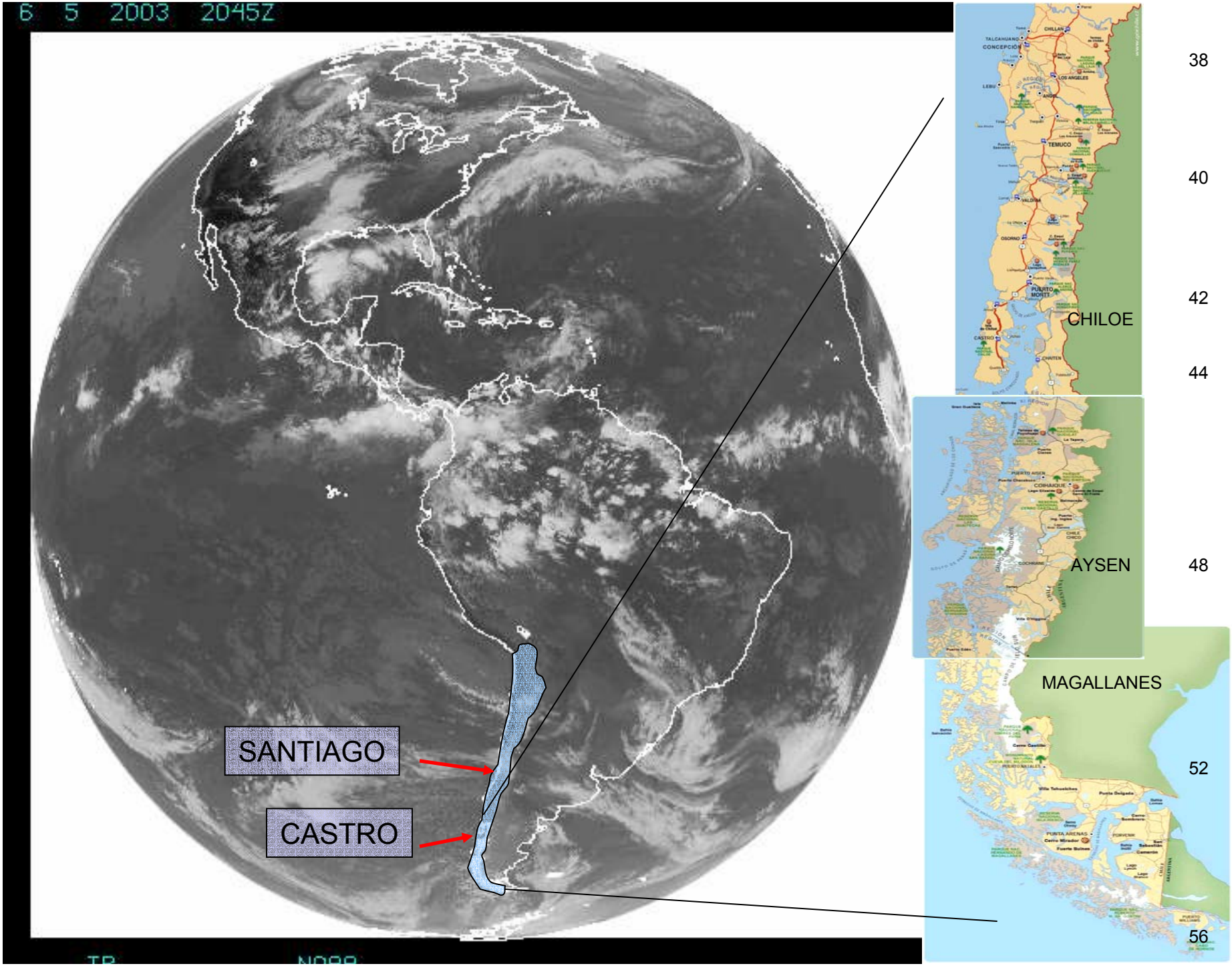
Recent PSP Outbreaks in Chile. Trends, Impacts and the Need for High-throughput Receptor Binding Assays

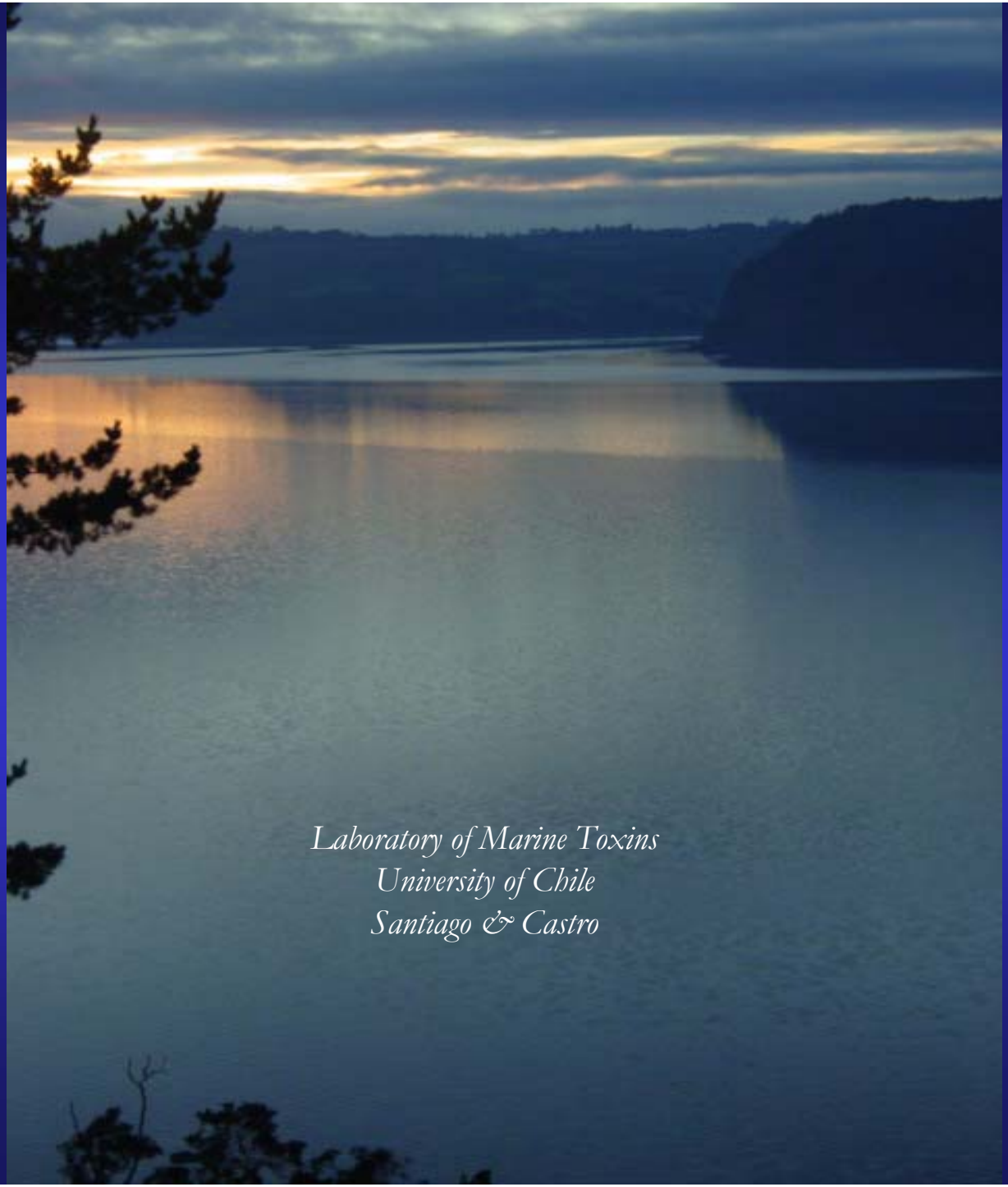
Benjamin A. SUAREZ-ISLA
Laboratory of Marine Toxins, Program of Physiology and Biophysics, ICBM
Faculty of Medicine
University of Chile, Santiago, Chile

Recent PSP outbreaks in Chile. Trends, impacts and the need for high-throughput receptor binding assays

Inter-Regional Workshop on Regulatory Practice and
the use of Receptor Binding Assays for Determination
of Harmful Algal Toxins in Seafood
1 - 5 September, 2003, ICTP - IAEA, Trieste, Italy

Benjamín A. Suárez-Isla
Laboratory of Marine Toxins
Program of Physiology and Biophysics, ICBM
Faculty of Medicine, University of Chile


6 5 2003 2045Z
SANTIAGO
CASTRO
CHILOE
AYSEN
MAGALLANES
38
40
42
44
48
52
56


Laboratory of Marine Toxins
University of Chile
Santiago & Castro

Laboratory of Marine Toxins
Program of Physiology and Biophysics
Institute of Biomedical Sciences
University of Chile
Santiago & Castro, Chiloé

National reference laboratory for marine toxins
Colaborating unit of the National Fisheries Service (SERNAPESCA) since 1995
FDA certified in July 2002
Certification analysis in all coastal areas in Chile. More than 95 customers
Over 10.000 samples analyzed in 2002

Research areas

Cellular physiology of toxic dinoflagelates
Interaction mechanisms between *A. Catenella* and marine bacteria
Development of functional assays for marine toxins

Consulting work

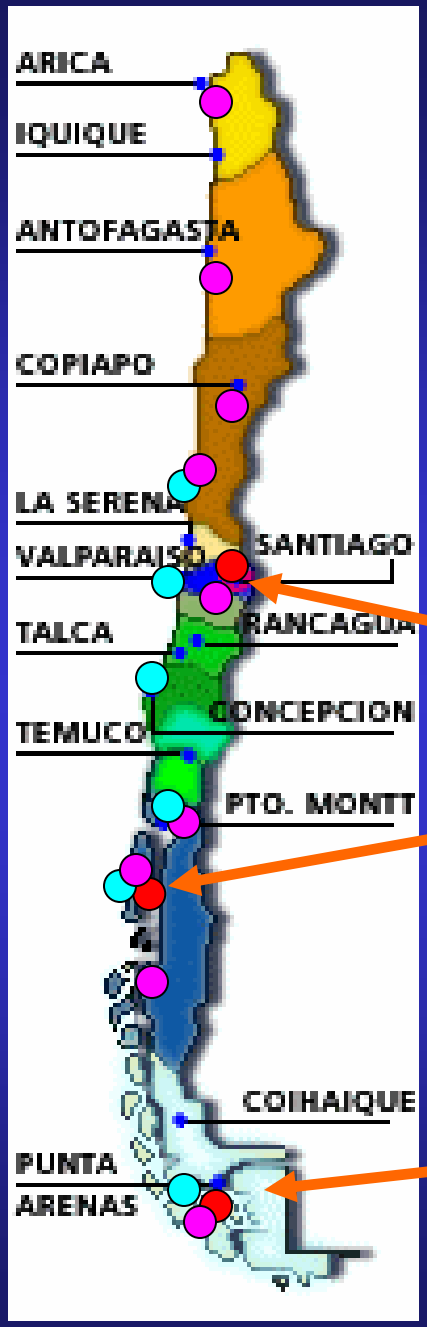
Installation of bioassays in processor plants
Heavy metal detoxification
Detoxification of marine toxins during canning process

Network of
certified laboratories

Regulatory Authorities

National Fisheries Services
(SERNAPESCA)
(Shellfish Sanitation Programmes
EU and USA)

Ministry of Health
Regional Health Services
(domestic consumption)



Toxin analysis

Phytoplankton
analysis

Regional Health
Services (PSP, DSP)

Laboratory of Marine Toxins
(Santiago; 1995)

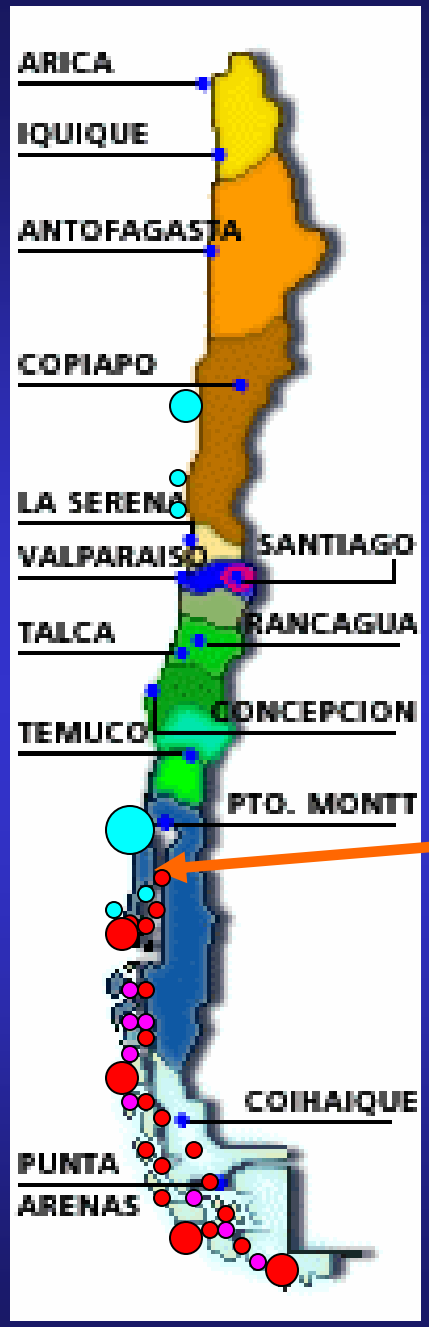
Castro (2002)

Instituto de Fomento
Pesquero (IFOP)

Distribution of marine toxins in Chile (1972 - 2002)

Laboratory of Marine Toxins University of Chile

IFOP Punta Arenas



Castro, Chiloé

ASP

DSP

PSP

Closed areas

Official methods of analysis applied in Chile

PSP: AOAC mouse bioassay.
Action level: 80 µg STX equivalent/100 grams of tissue

DSP: mouse bioassay.
A sample is considered positive if at least 2 of 3 injected mice die within 24 hours

VAM: HPLC with UV detection.
Action level: 20 µg/g.

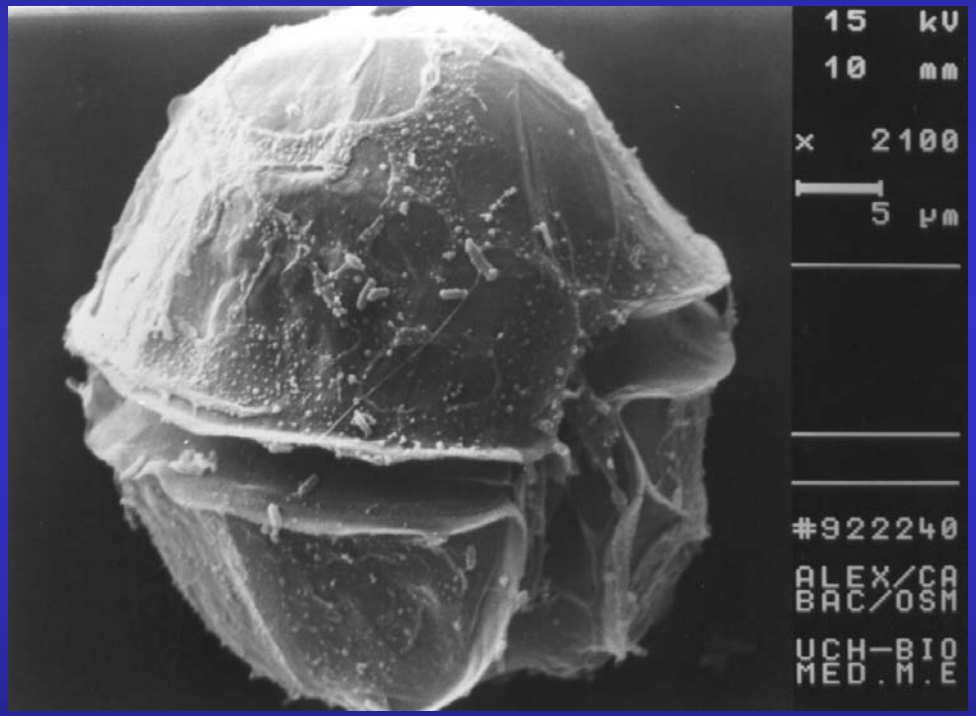
Method performance is regularly checked by the Instituto de Salud Pública, Ministry of Health.

OUTBREAKS OF PARALYTIC SHELLFISH POISON IN CHILE

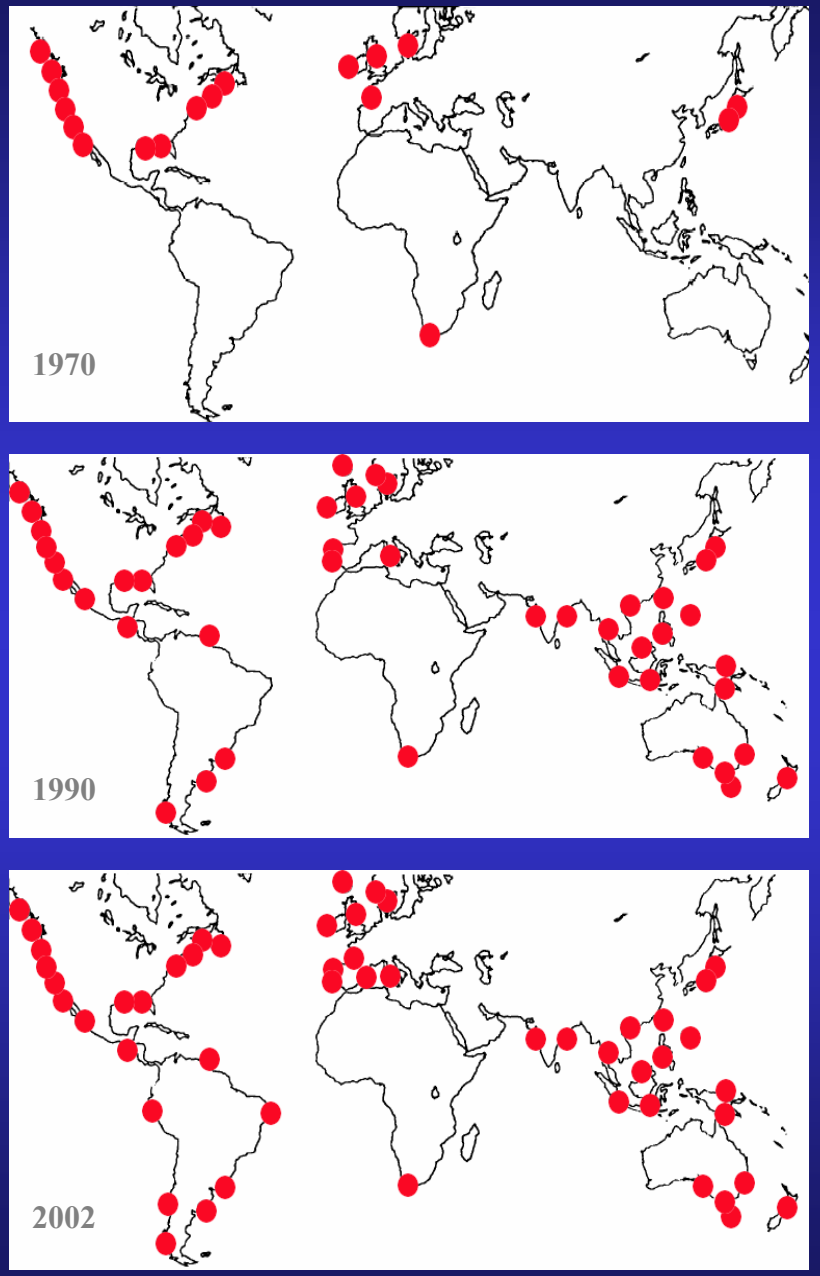
Data from:

Instituto de Fomento Pesquero (Dr. Leonardo Guzmán)
Laboratorio Plancton Andino (Dr. Alejandro Clément)
Regional Health Services (Dr. Ramón Andrade)
National Fisheries Services
Laboratory of Marine Toxins

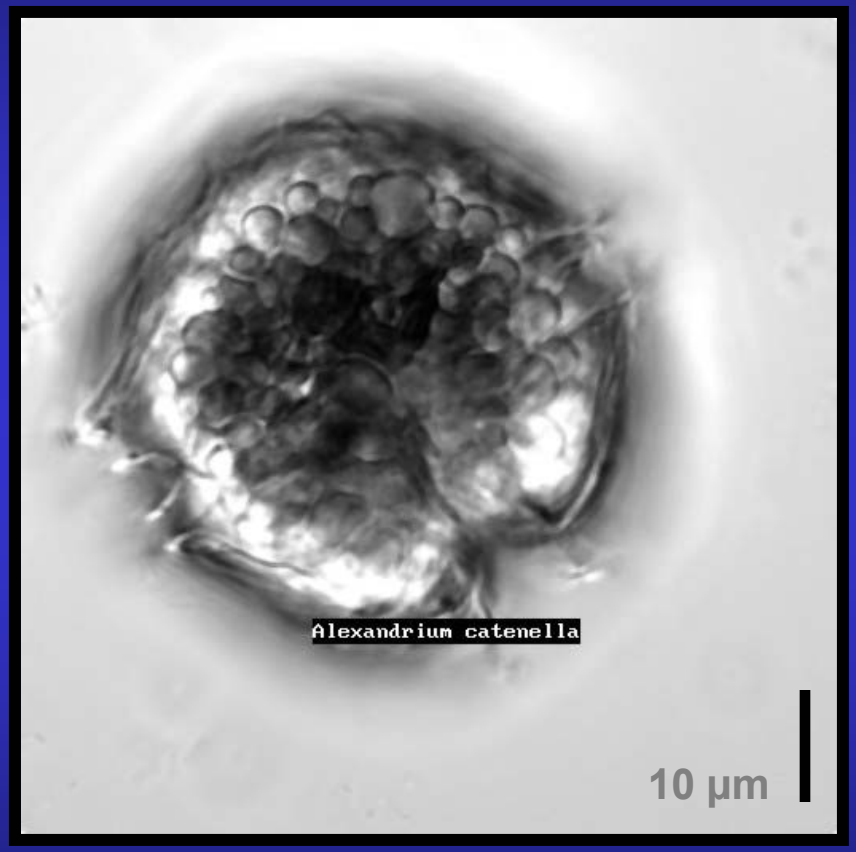
Changes in PSP distribution in three decades

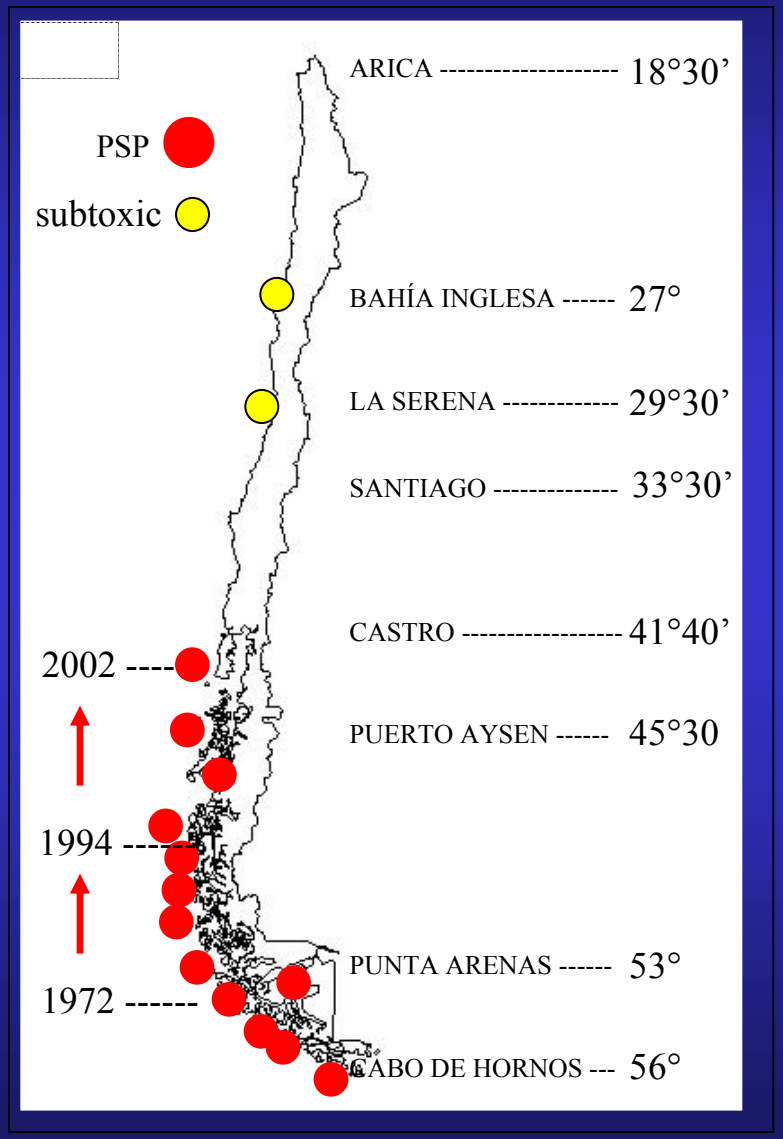


Alexandrium catenella, clone ACC01, Aysén fjord; Amaro et al.,



Changes in PSP distribution in Chile since 1972

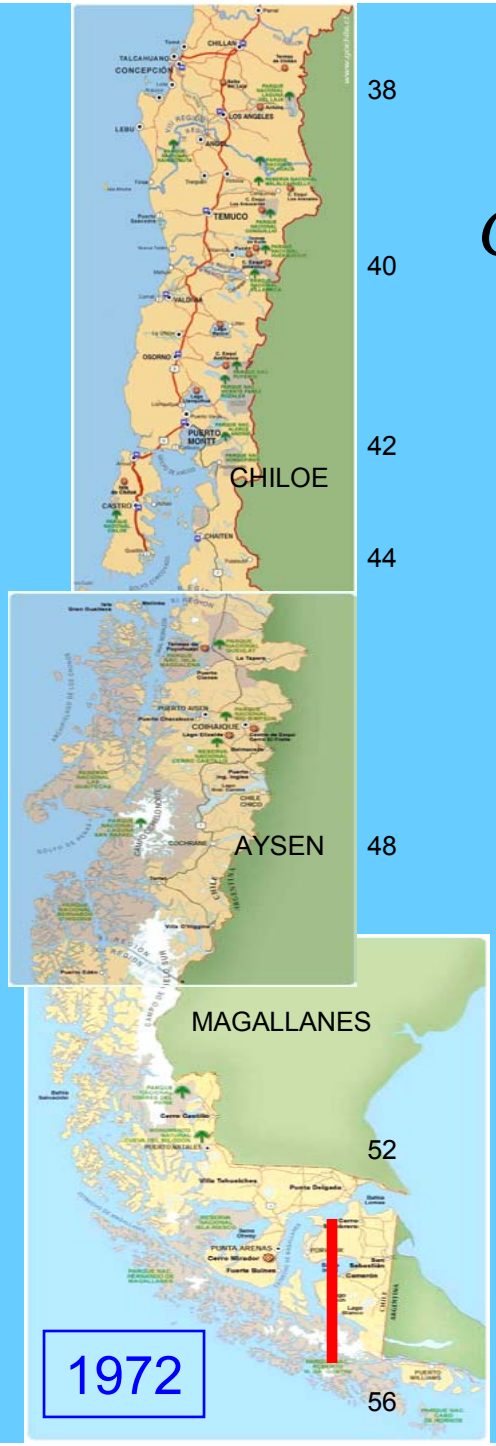


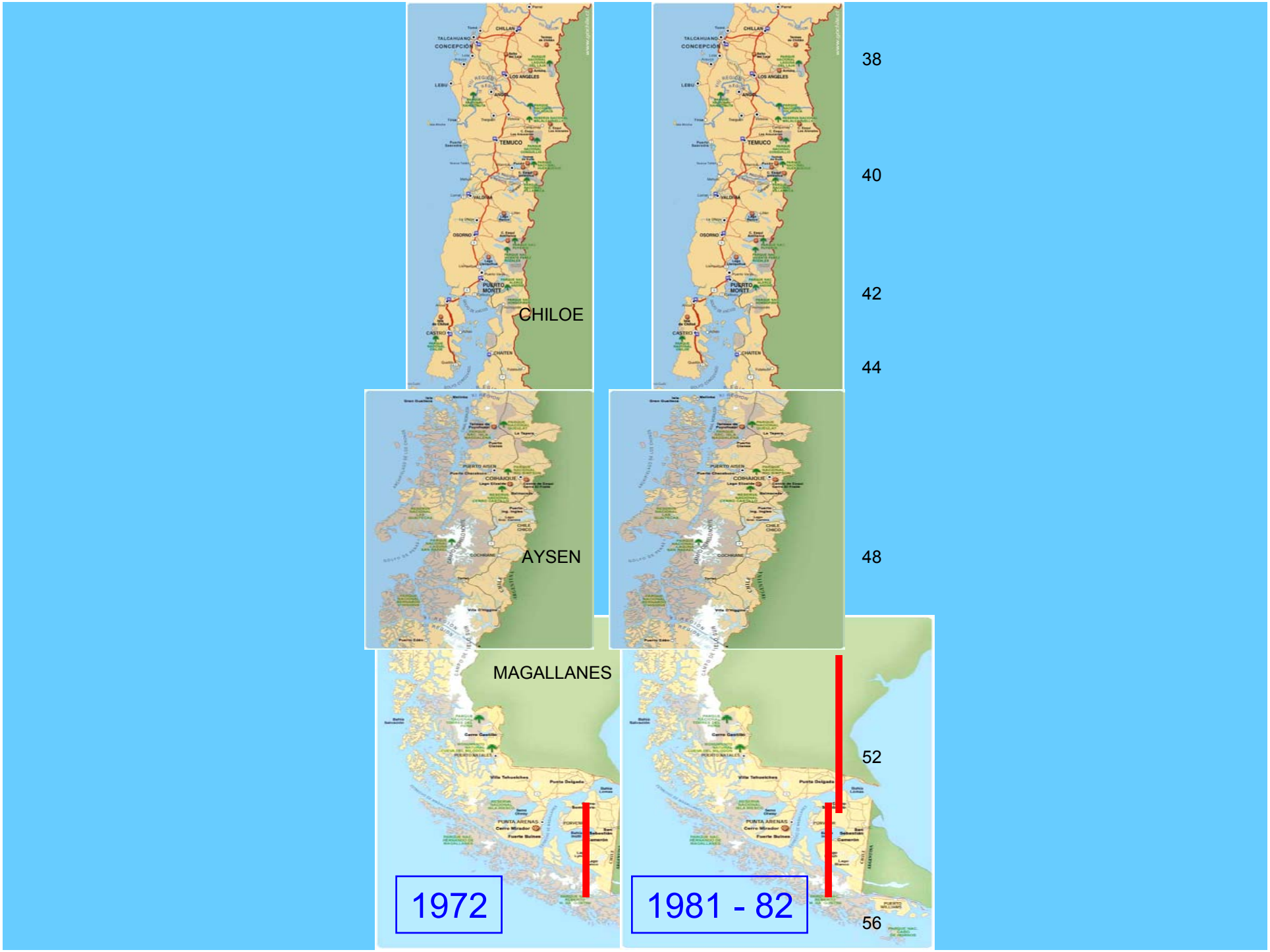


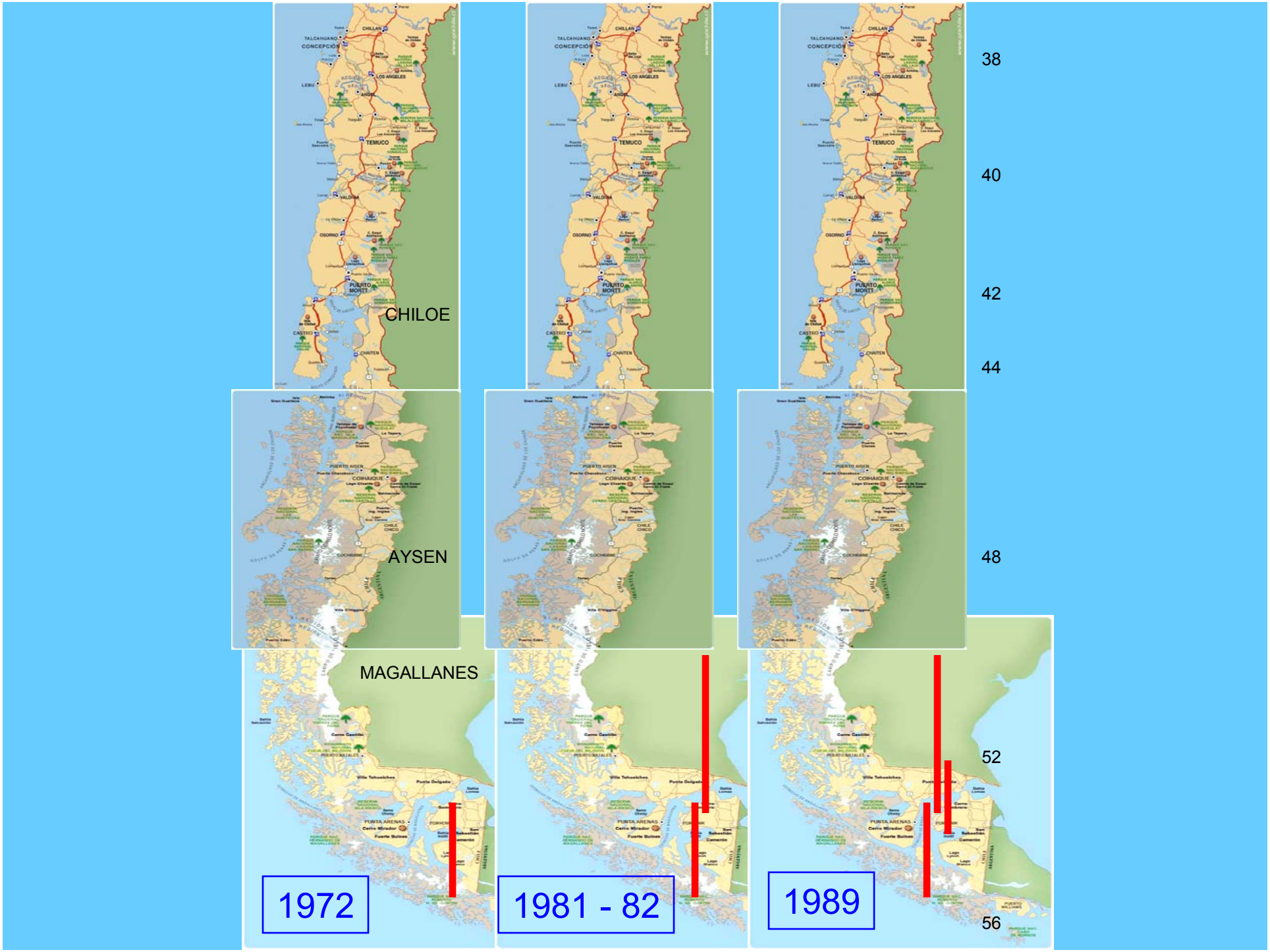
Suárez-Isla et al. "Impacto económico de las floraciones de microalgas nocivas en Chile y datos recientes sobre la ocurrencia de Veneno Amnésico de los Mariscos" Floraciones Algales Nocivas en el Cono Sur Americano. Monografías del Instituto Español de Oceanografía, Vigo (2002).

Changes in PSP distribution in Chile since 1972

(modified from Guzmán et al., 2002)




38
40
42
44
48
52
56
CHILOE
AYSEN
MAGALLANES
1972
1981 - 82


CHILOE
AYSEN
MAGALLANES
38
40
42
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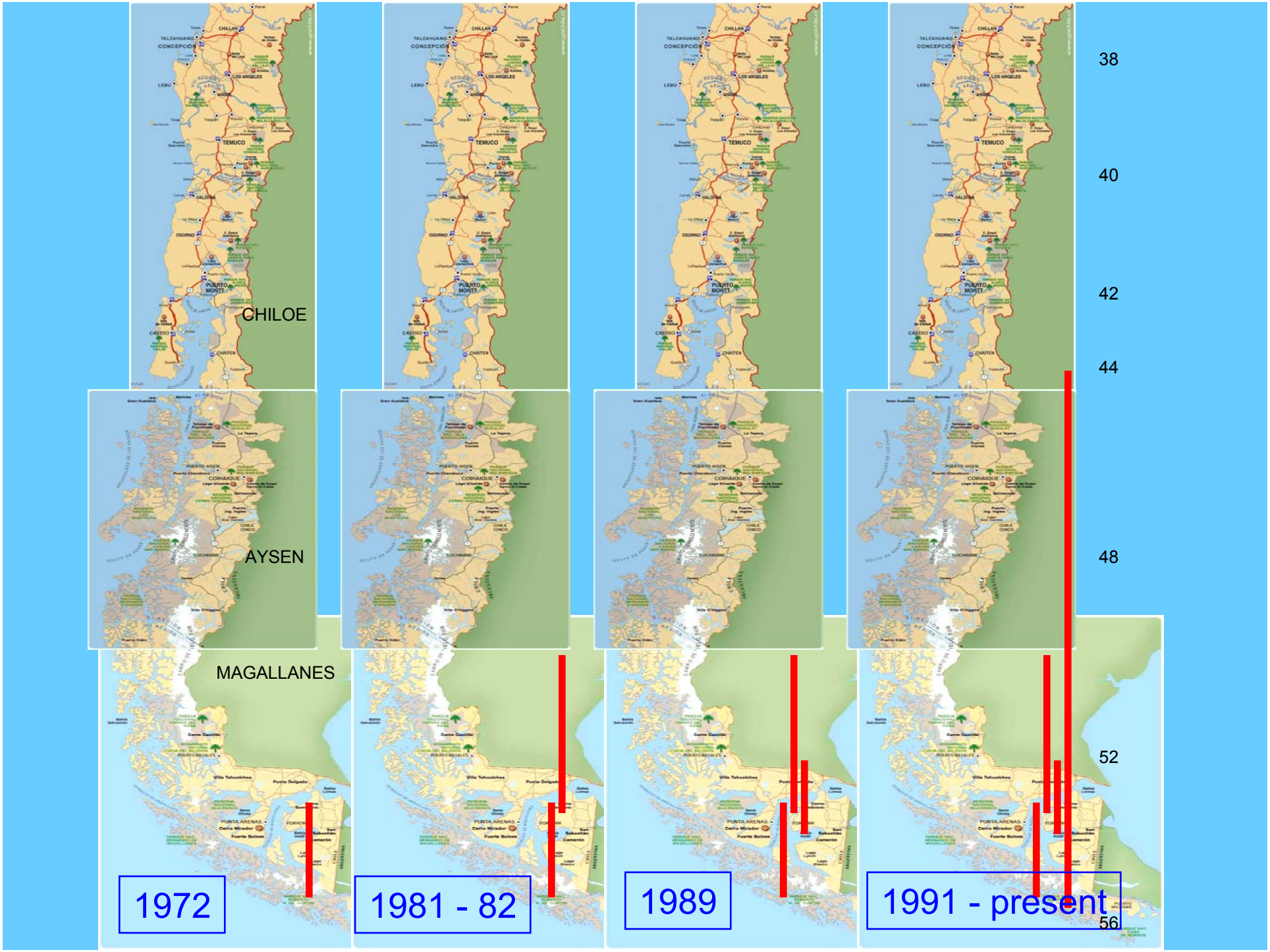
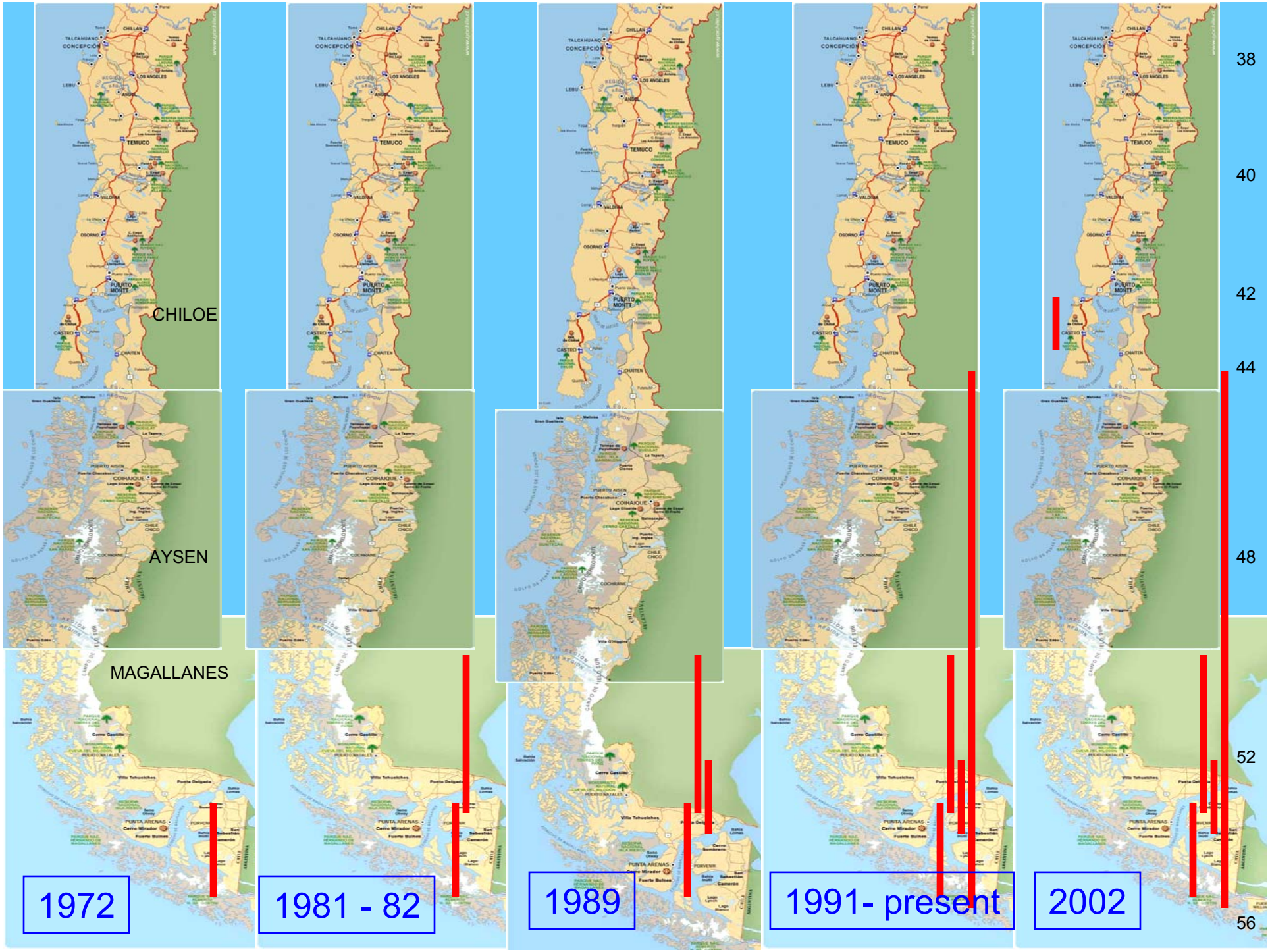
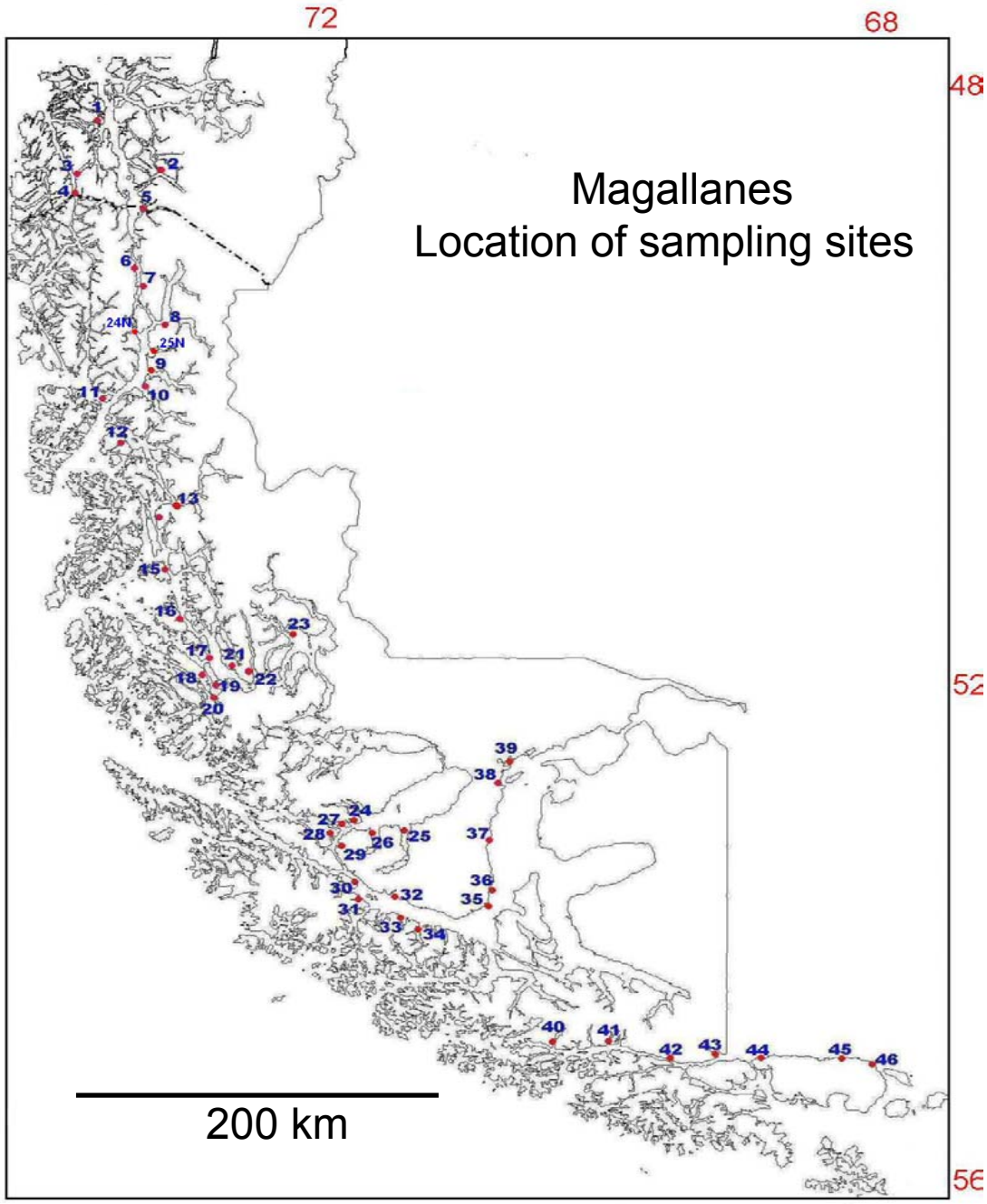
48
52
56
1972
1981 - 82
1989


CHILOE
AYSEN
MAGALLANES
38
40
42
44
48
52
56
1972
1981 - 82
1989
1991 - present


CHILOE
AYSEN
MAGALLANES
1972
1981 - 82
1989
1991- present
2002
38
40
42
44
48
52
56


72
68
48
52
56
Magallanes
Location of sampling sites
1
2
3
4
5
6
7
8
24N
25N
9
10
11
12
13
15
16
17
18
19
20
21
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23
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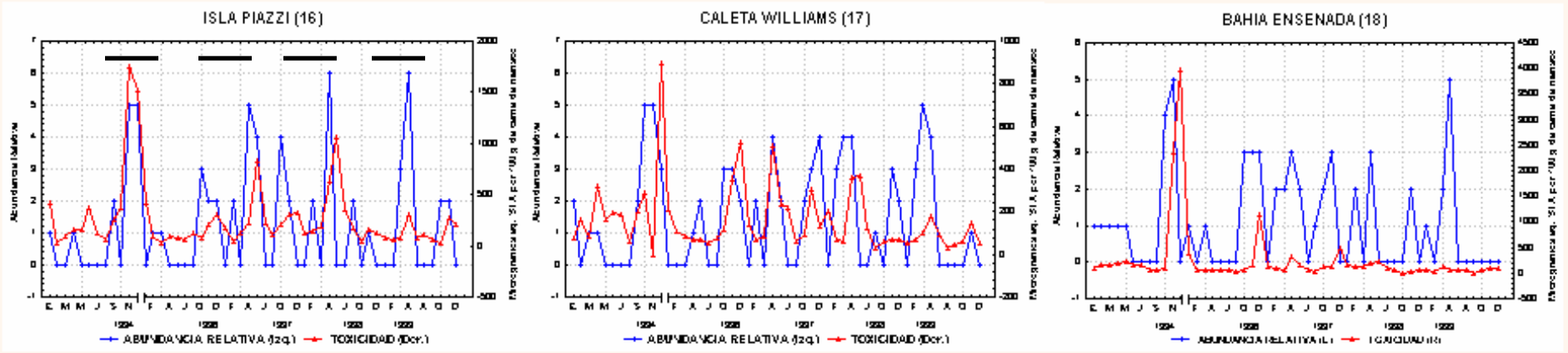
37
38
39
40
41
42
43
44
45
46
200 km

PSP TOXICITY AND RELATIVE ABUNDANCE OF *A. catenella*



TIME SERIES 1994 - 1999 IN SELECTED AREAS FROM MAGALLANES

Data from Guzmán et al., 2002. Instituto de Fomento Pesquero, Punta Arenas

IMPACTS ON PUBLIC HEALTH IN CHILE

Paralytic Shellfish Poison (PSP) (saxitoxins)
Main causative species: *Alexandrium catenella*

32 deaths and 421 intoxicated persons since 1972

3 deaths and 73 intoxicated only between January and June 2002 (Chiloé)
3 deaths and 9 intoxicated in Magallanes in June 2002

Diarrhetic Shellfish Poisoning (DSP) (okadaic acid, yessotoxins, pectenotoxins)
Causative species: *Dinophysis acuta.* Over 800 reported cases

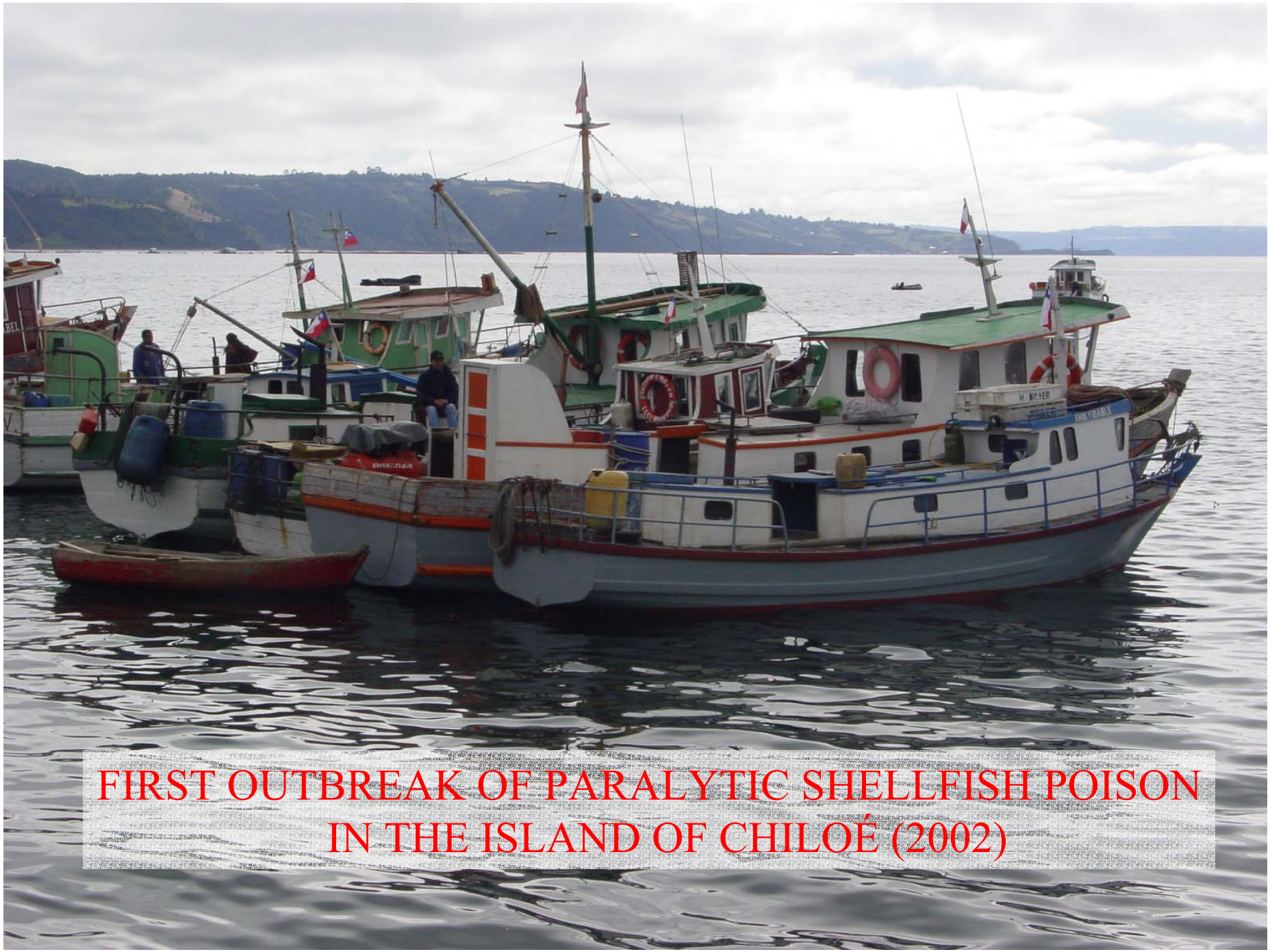
Amnesic Shellfish Poison (ASP) (domoic acid)
Causative species: diatoms *Pseudonitzschia spp.* No reported cases

Laboratorio de Toxinas Marinas
Facultad de Medicina
Universidad de Chile

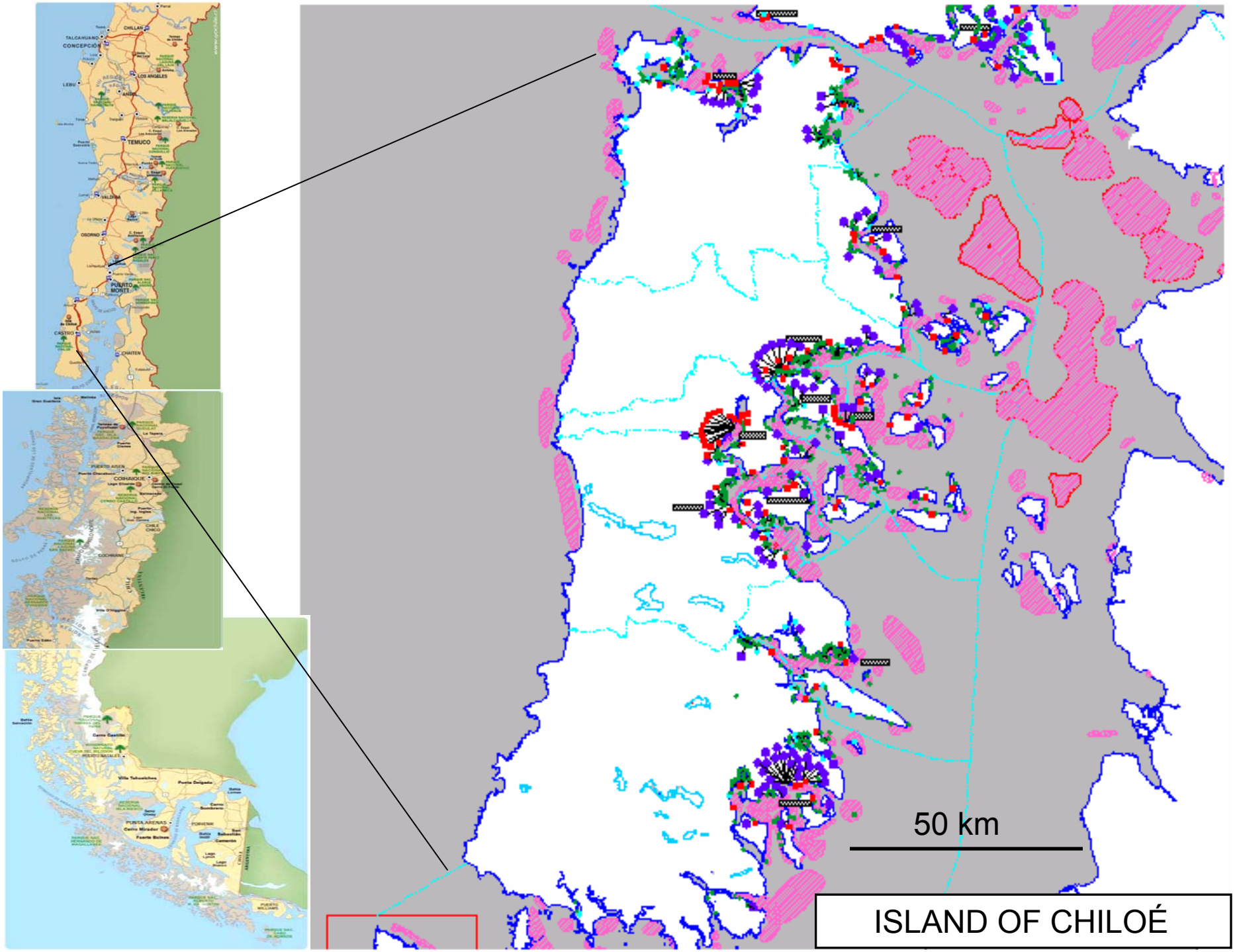
Cases of PSP intoxications in Chile (1972 - 2003)						
CASE	**Year**	**Mo**	**No of patients**	**Deaths**	**Location**	**shellfish**
1	1972	10	3	3	Bahía Bell	cholga
2	1981	2	26	2	Seno Unión	cholga
3	1989	4	8	0	Estero Núñez	cholga
4	1991	3	95	2	Bahía Nash	chorito
5	1991	11	125	2	Seno Unión ?	cholga
6	1991	12	2	1	Seno Nevado	cholga
7	1992	1	14	6	Bahía Woodsworth	chorito
8	1992	1	5	0	Estero Asia	chorito
9	1992	2	3	0	Km 49 Sur	chorito
10	1992	3	1	1	Caleta La Olla	nd
11	1992	5	3	0	Paso Nuevo	cholga
12	1992	7	1	0	Isla Vancouver	cholga
13	1992	7	3	1	Paso Schoal	cholga
14	1992	12	6	0	Puerto Williams	chorito
15	1994	1	8	0	San Juan	chorito
16	1994	1	1	0	Los Ñires	chorito
17	1994	1	2	1	Punta Arenas	chorito
18	1994	1	1	0	Punta Arenas	chorito
19	1994	2	1	0	Punta Arenas	chorito
20	1994	4	1	0	Bahía Gente Grande	cholga
21	1994	6	1	1	Seno Ringove	cholga
22	1995	1	4	0	Chabunco	chorito
23	1995	2	1	0	Seno Profundo	cholga
24	1995	5	14	1	Isla Toto, Aysén	cholga
25	1996	?	21	2	Aysén	nd

(Cholga: *Aulacomya ater* . Chorito: *Mytilus chilensis* . Caracol piquilhue: *Adelomelon ancilla*)

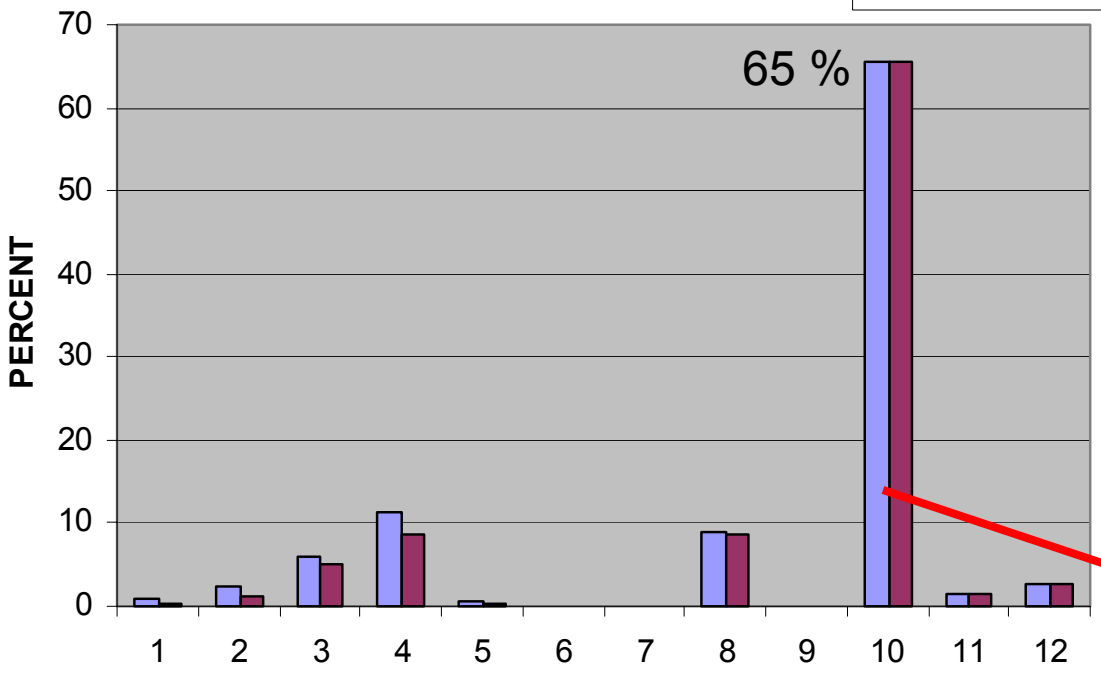
Cases of PSP intoxications in Chile (1972 - 2003)						
CASE	**Year**	**Mo**	**No of patients**	**Deaths**	**Location**	**shellfish**
26	1997	?	1	0	Aysén	nd
27	1997	1	2	0	Isla Isabel	cholga
28	1997	1	1	0	Fuerte Bulnes	cholga
29	1997	1	1	0	Santa María	cholga
30	1998	2	9	1	Aysén	nd
31	1998	3	10	1	Aysén	cholga
32	1998	4	1	0	Aysén	cholga
33	1998	?	2	1	Aysén	cholga
34	1998	11	2	0	Canal Magdalena	caracol
35	1999	3	1	0	Aysén	cholga
36	2000	3	6	0	Aysén	cholga
37	2000	11	1	0	Isla Lucía	cholga
	2001		0	0		
38	2002	1	3	0	Quellón	cholgas
39	2002	1	11	0	Quellón	cholgas
40	2002	1	29	0	Mehuin (X Region)	nd
41	2002	1	1	0	Castro	choros
42	2002	3	2	1	Isla Tranqui	cholgas (*)
43	2002	3	3	0	Isla Acui	cholgas (*)
44	2002	3	3	0	Huillinco	cholgas (*)
45	2002	3	4	0	Quellón	cholgas
46	2002	4	4	0	Yaldad	choritos
47	2002	6	9	3	Canal Beagle (XII)	cholgas
	2003		0	0		
TOTALS			**456**	**30**		
(*)	collected from salmon nets					
(Cholga: *Aulacomya ater* . Chorito: *Mytilus chilensis* . Caracol piquilhue: *Adelomelon ancilla*)						

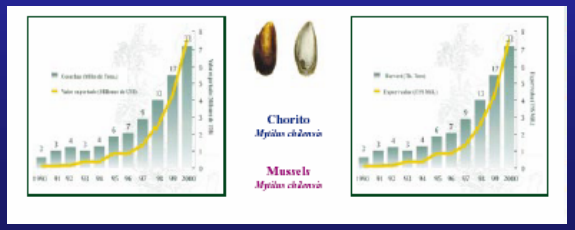


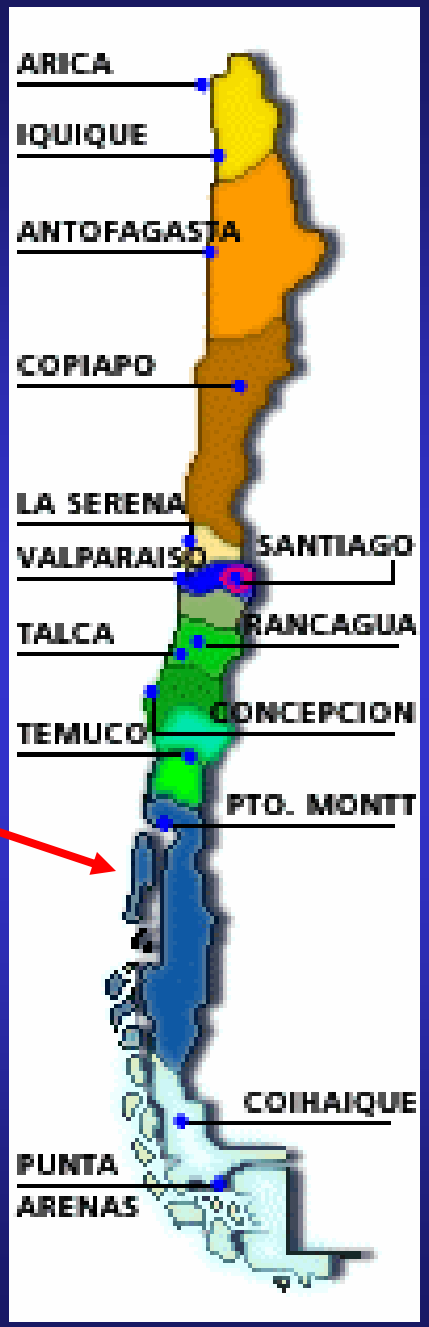
FIRST OUTBREAK OF PARALYTIC SHELLFISH POISON IN THE ISLAND OF CHILOÉ (2002)

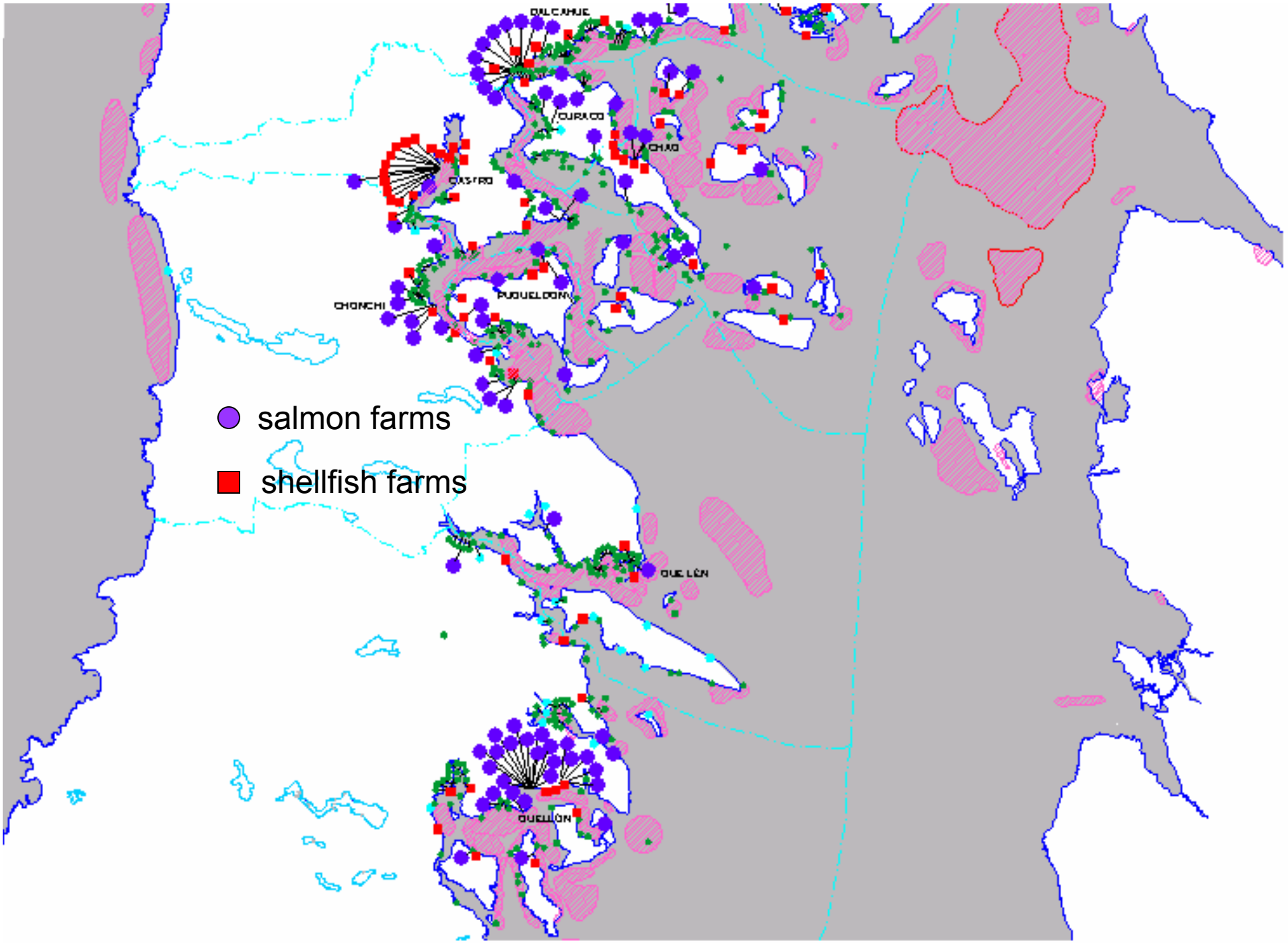

50 km
ISLAND OF CHILOÉ

DISTRIBUTION OF SHELLFISH PRODUCTION PER REGION

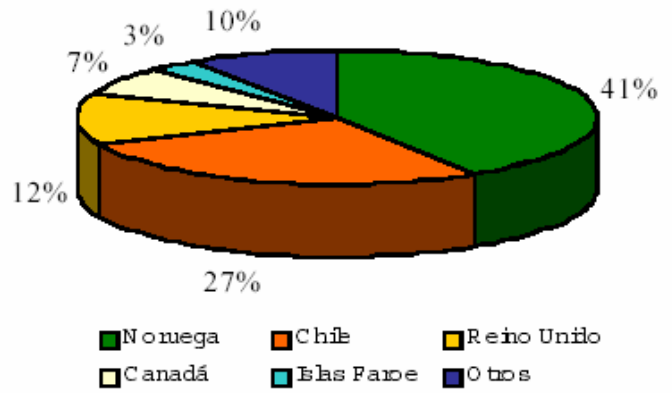






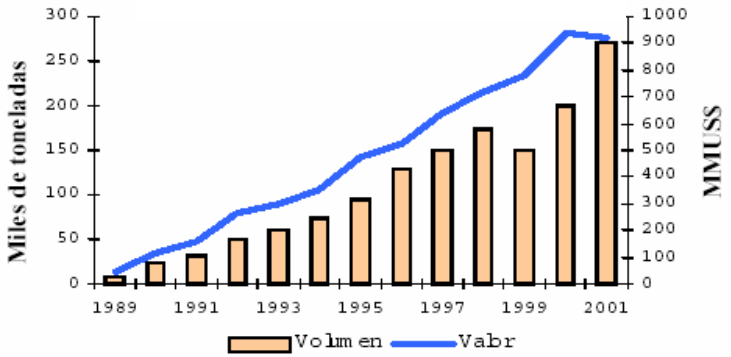

salmon farms
shellfish farms

Salmon producing countries (2000)

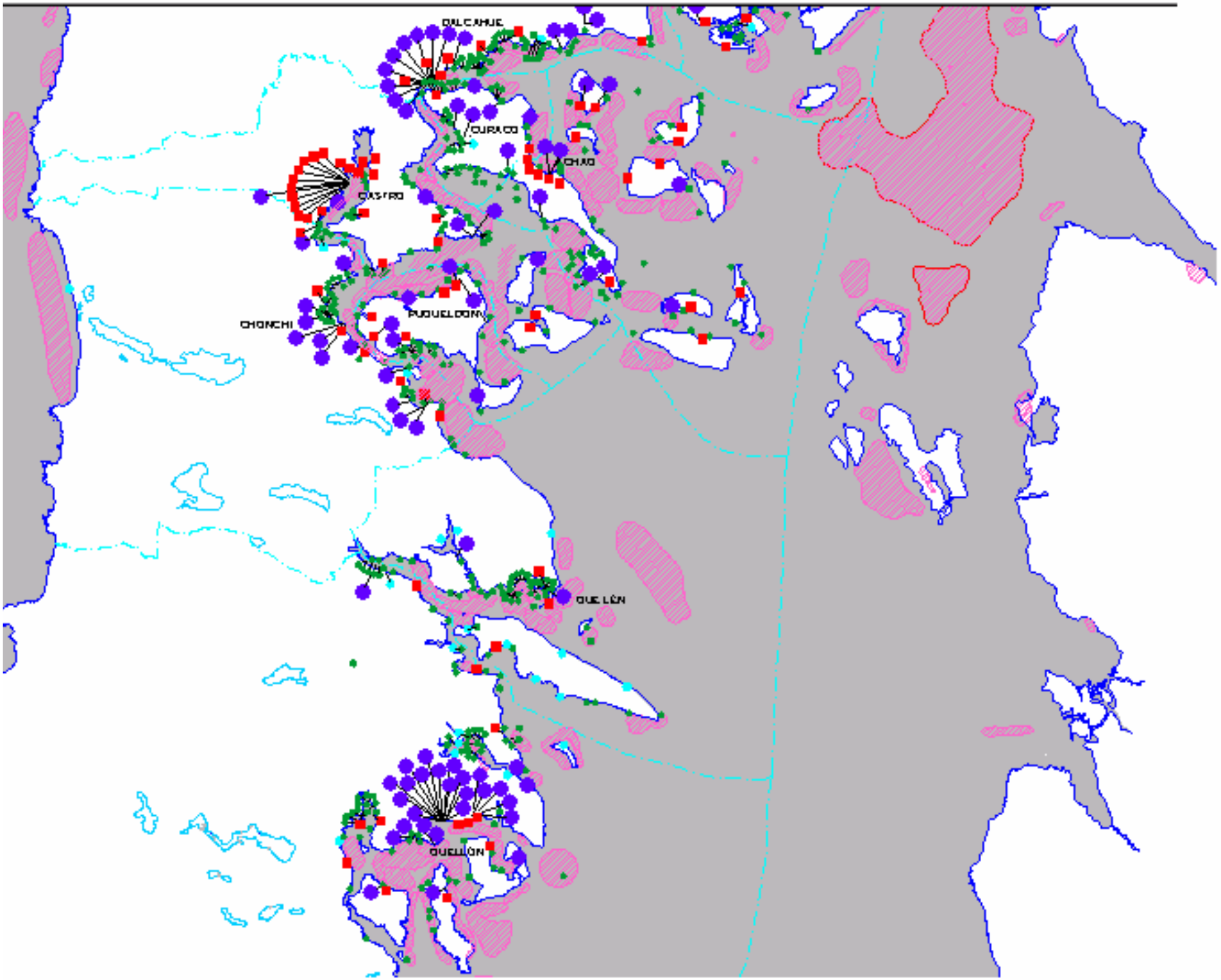


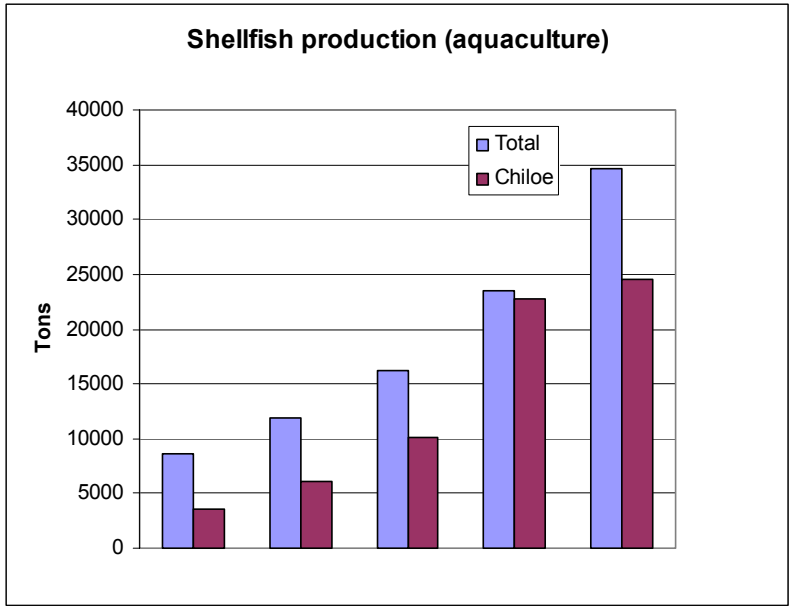
Fuente: Asociación de Productores de Salmón y Trucha

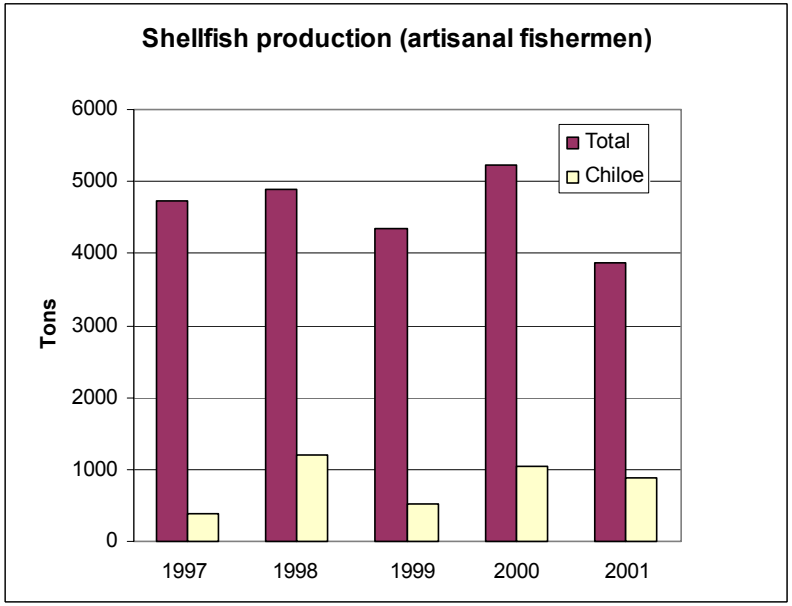
Exports salmon & trout

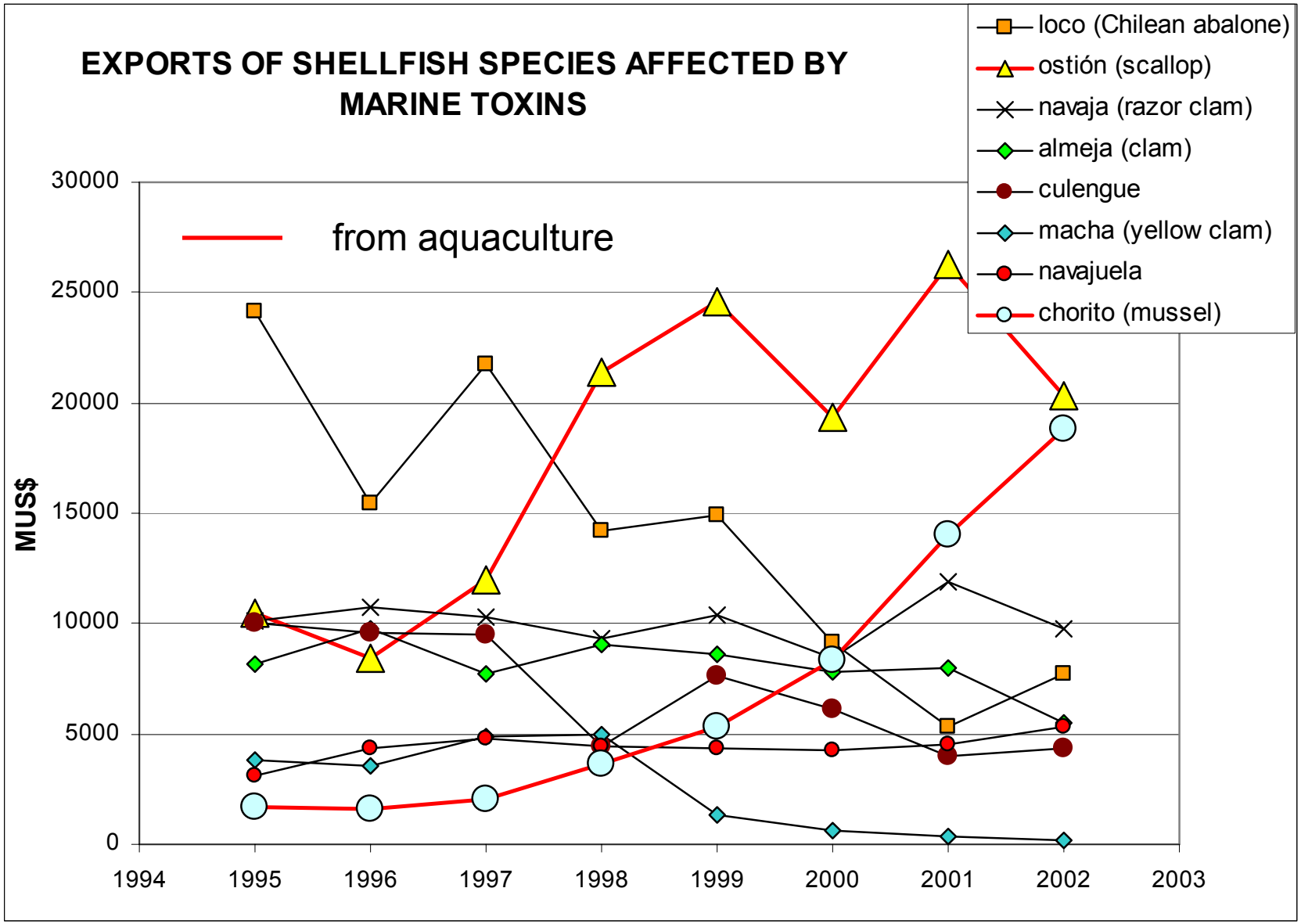


Fuente: Banco Central


DALCAHUE
CURACO
CHAO
CASTRO
CHONCHI
PUQUELDON
QUELÓN
QUELLÓN


Shellfish production (aquaculture)
Tons
40000
35000
30000
25000
20000
15000
10000
5000
0
Total
Chiloe


Shellfish production (artisanal fishermen)
Tons
6000
5000
4000
3000
2000
1000
0
Total
Chiloe
1997
1998
1999
2000
2001


EXPORTS OF SHELLFISH SPECIES AFFECTED BY MARINE TOXINS
from aquaculture
MUS$
30000
25000
20000
15000
10000
5000
0
1994
1995
1996
1997
1998
1999
2000
2001
2002
2003
loco (Chilean abalone)
ostión (scallop)
navaja (razor clam)
almeja (clam)
culengue
macha (yellow clam)
navajuela
chorito (mussel)

SAMPLING SITE	ug STX /100 g	Date
Isla Laitec	61	Jan 22
Quellon	34	Jan 28
Isla Quilan (Guapiquilan)	227	Jan 31
Punta Yelcho	82	March 3
Quellon Viejo	112	March 5
Isla Cailin	195	March 15
Queilen	52	March 21
Detico	1744	March 21
Terao	728	March 27
Teupa (Chonchi)	55	March 27
Teupa SS	977	April 1
Teupa (Plaza)	233	April 1
Vilupulli	305	April 1
Chullec	292	April 4
Huenao	122	April 4
Luicura	103	April 11
Palqui	153	April 25
Calen	0	

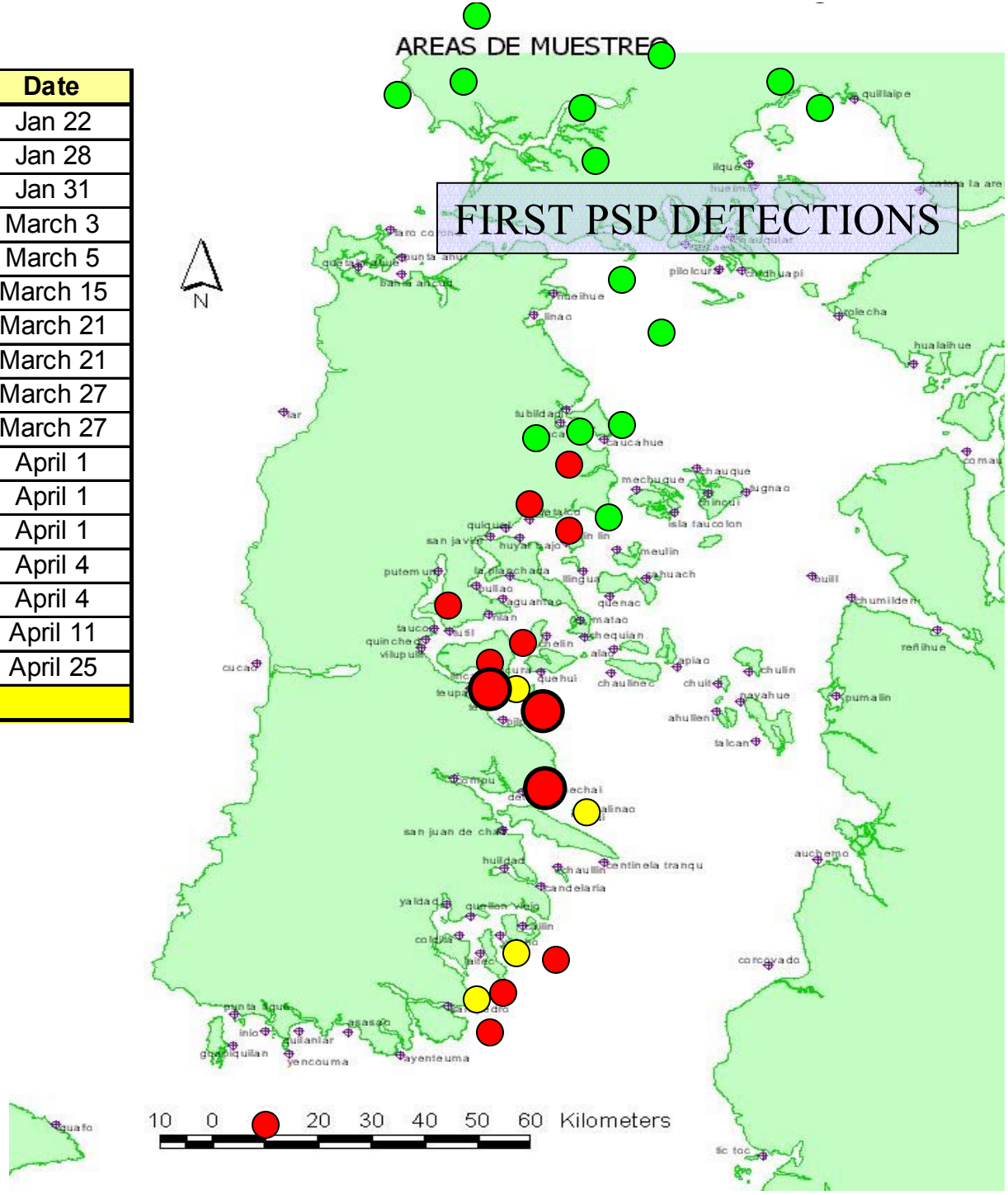
Negative

< 80 µg/100 g

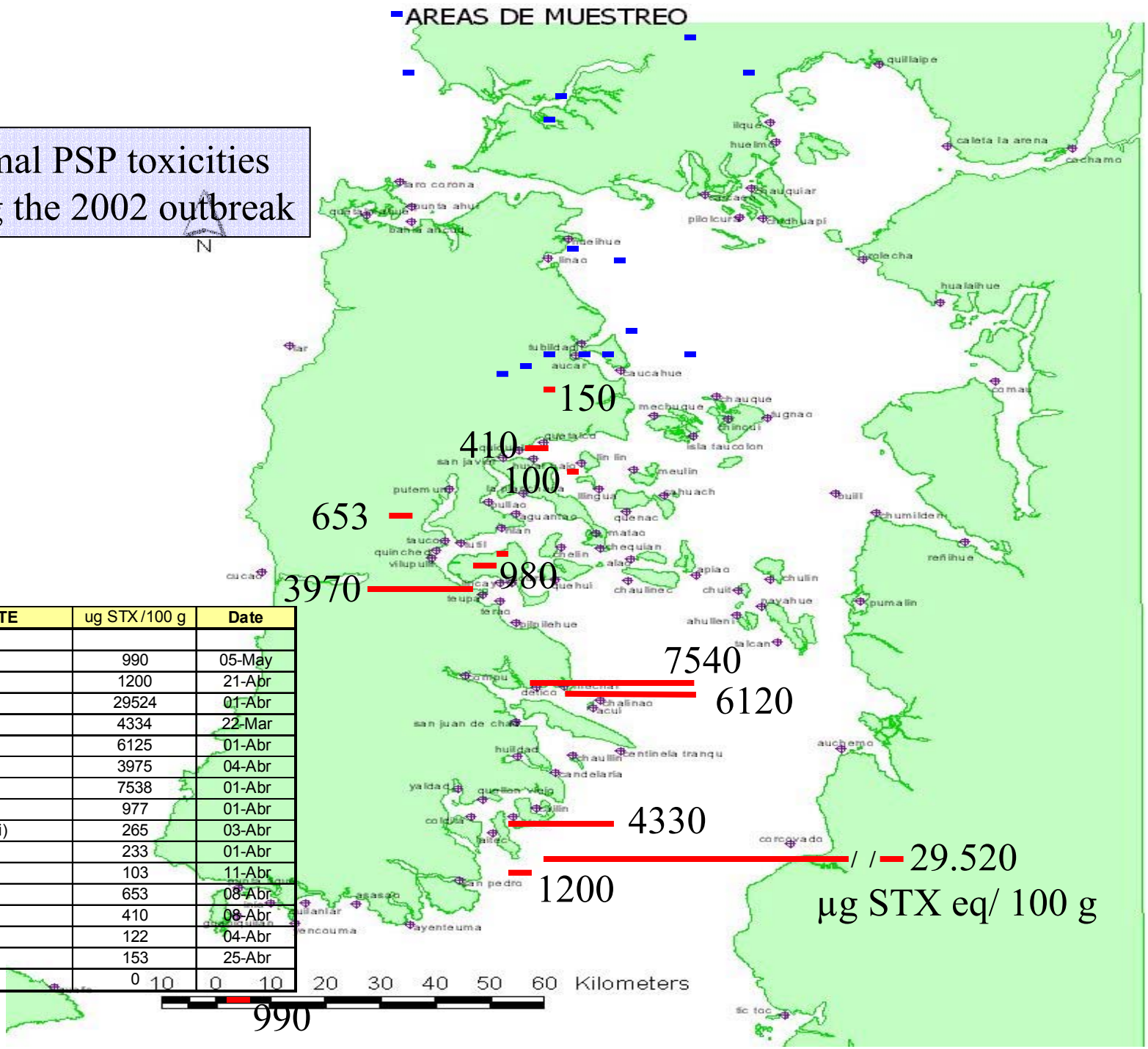
80 - 500

500 - 2000

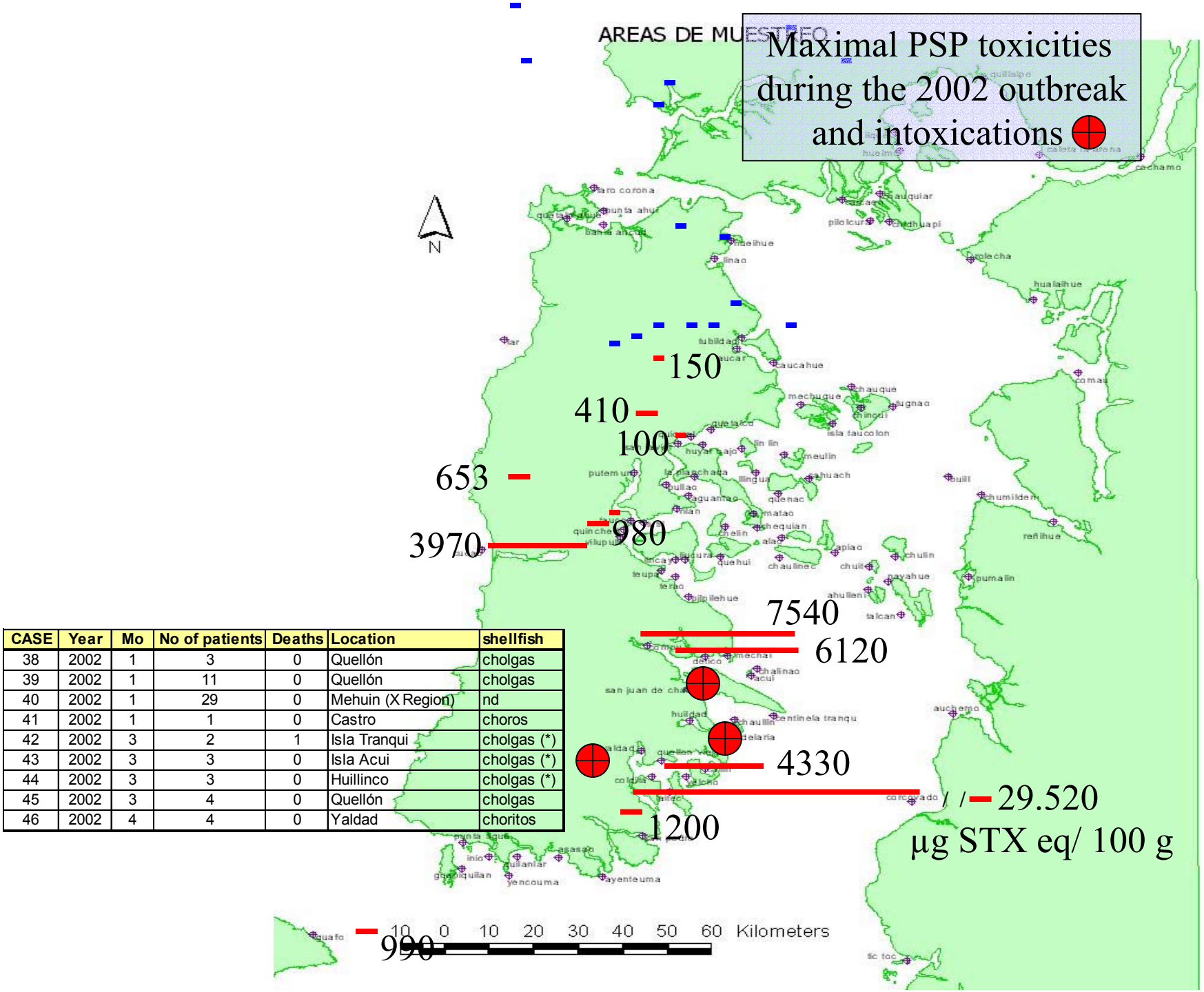
> 2000



Maximal PSP toxicities during the 2002 outbreak

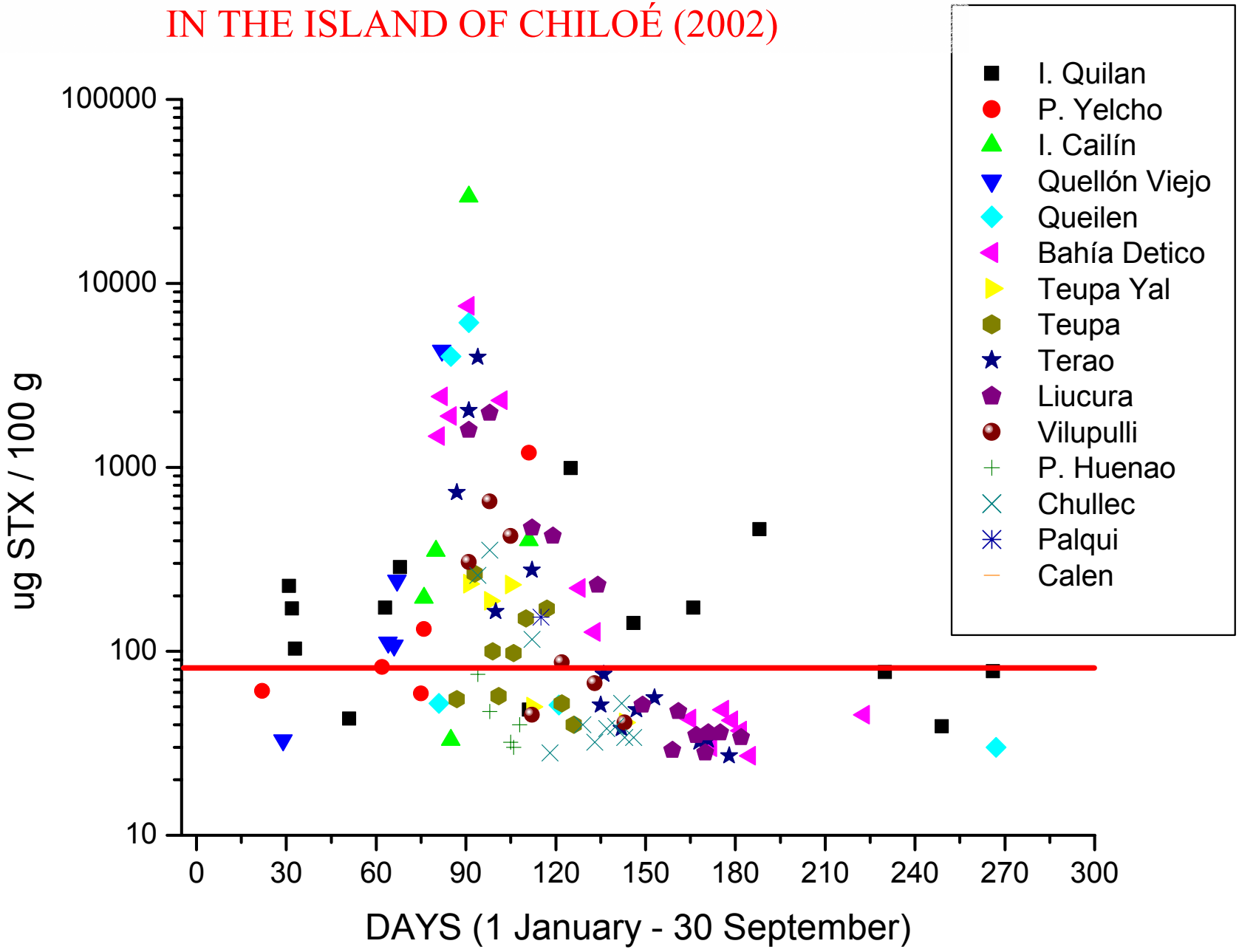


SAMPLING SITE	ug STX/100 g	Date
Isla Quilan	990	05-May
Punta Yelcho	1200	21-Abr
Isla Cailin	29524	01-Abr
Quellon Viejo	4334	22-Mar
Queilen	6125	01-Abr
Terao	3975	04-Abr
Detico	7538	01-Abr
Teupa SS	977	01-Abr
Teupa (Chonchi)	265	03-Abr
Teupa (Plaza)	233	01-Abr
Luicura	103	11-Abr
Vilupulli	653	08-Abr
Chullec	410	08-Abr
Huenao	122	04-Abr
Palqui	153	25-Abr
Calen	0	

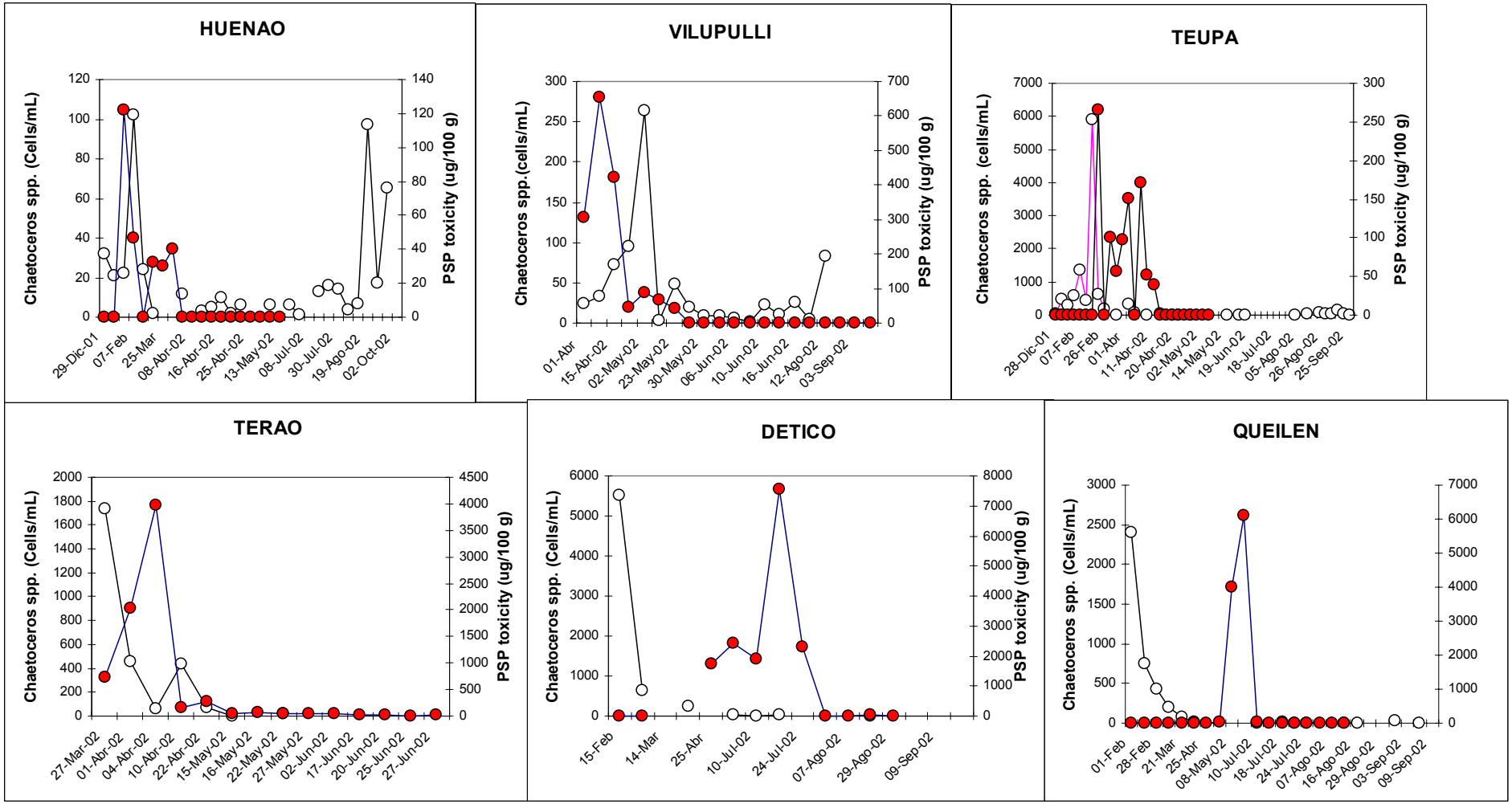


CASE	Year	Mo	No of patients	Deaths	Location	shellfish
38	2002	1	3	0	Quellón	cholgas
39	2002	1	11	0	Quellón	cholgas
40	2002	1	29	0	Mehuin (X Region)	nd
41	2002	1	1	0	Castro	choros
42	2002	3	2	1	Isla Tranqui	cholgas (*)
43	2002	3	3	0	Isla Acui	cholgas (*)
44	2002	3	3	0	Huillinco	cholgas (*)
45	2002	3	4	0	Quellón	cholgas
46	2002	4	4	0	Yaldad	choritos

FIRST OUTBREAK OF PARALYTIC SHELLFISH POISON IN THE ISLAND OF CHILOÉ (2002)



Diatom blooms and PSP toxicity



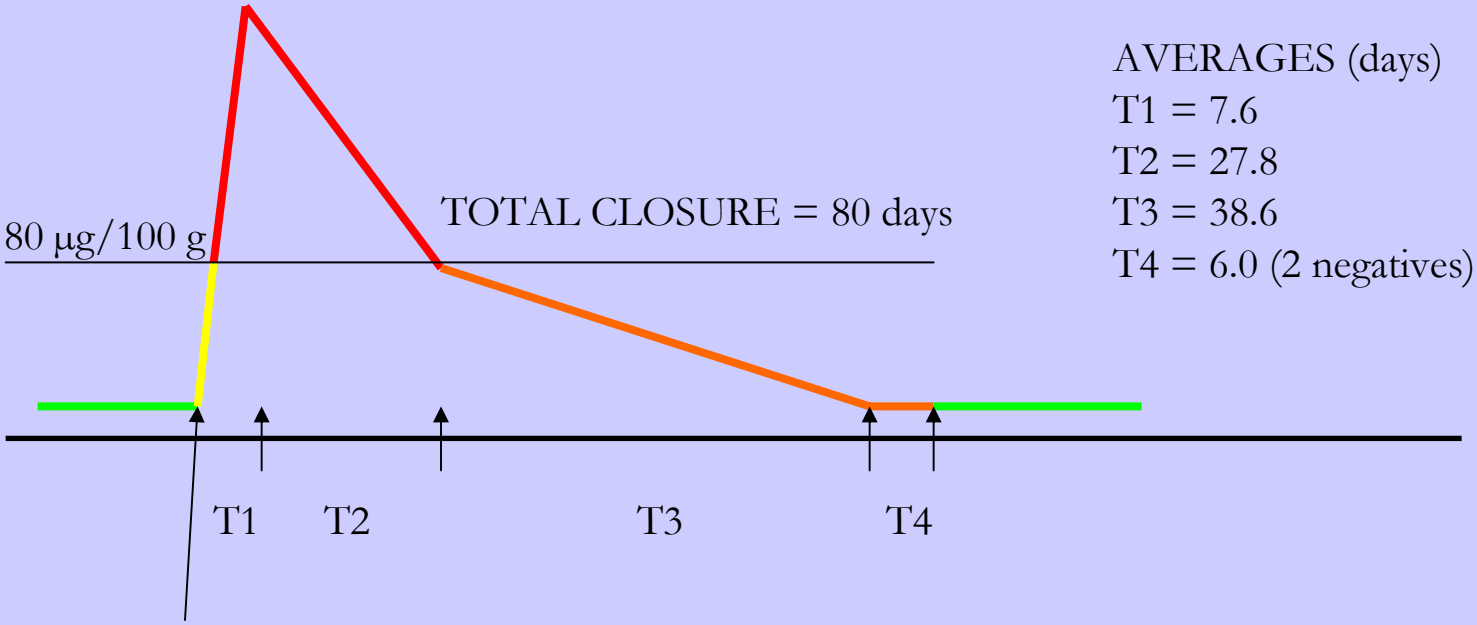
PSP toxicity

***Chaetoceros* spp.**

DURATION OF TOXIC AND Y SUBTOXIC PERIODS IN ACQUACULTURE SITES (2002 PSP OUTBREAK)

LOCATION	maximal toxicity	T1	T2	T3	T4	TOTAL
	(µg/100 g)		> 80 µg	< 80 µg	2 negatives	days
Huenao	75	3	4	14	6	27
Chullec	355	7	22	28	6	63
Liucura	1967	12	43	26	6	87
Vilupulli	653	12	29	28	6	75
Teupa Plaza	233	4	19	8	6	37
Teupa Yal	265	1	27	9	6	43
Terao	3975		29	53	6	88
Detico	7538	14	49	143	6	212
Queilén	6125	13	30	34	6	83
	TOTAL DAYS	**66**	**252**	**343**	**54**	**715**
	average	7,57	27,75	38,63	6,00	**79,95**
	S.D:	5,12	13,07	41,72		54,64
	%	9,47	34,71	48,31	7,51	

MODEL OF THE INTOXICATION-DETOXIFICATION PROCESS
PSP OUTBREAK CHILOÉ 2002

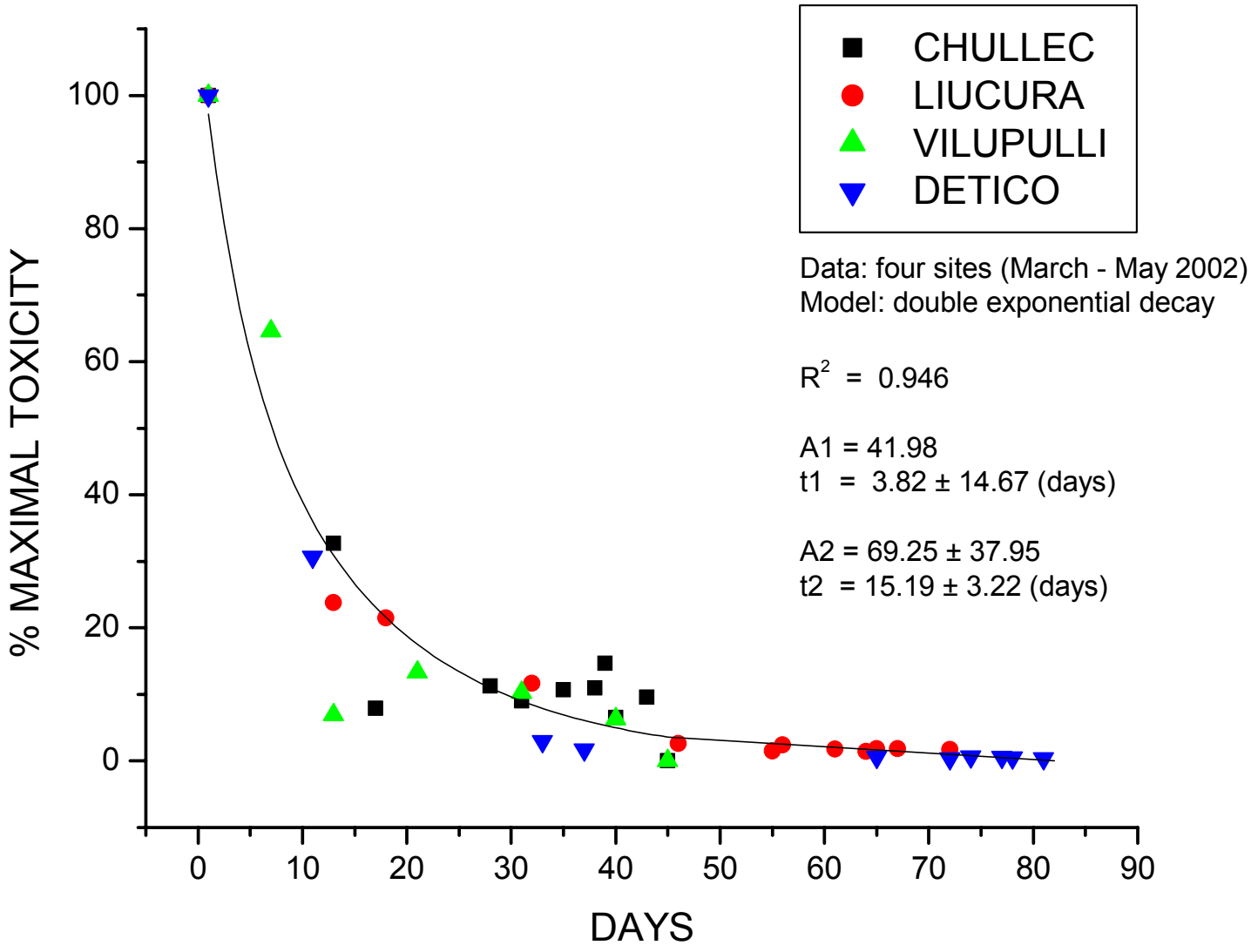


AVERAGES (days)
T1 = 7.6
T2 = 27.8
T3 = 38.6
T4 = 6.0 (2 negatives)

	T1	T2	T3	T4	TOTAL
		> 80 µg	< 80 µg	2 negatives	days
TOTAL DAYS	**66,00**	**252,00**	**343,00**	**54,00**	**715,00**
average	7,57	27,75	38,63	6,00	**79,95**
S.D:	5,12	13,07	41,72		54,64
%	9,47	34,71	48,31	7,51	

DETOXIFICATION OF PSP TOXINS IN MUSSELS

(*Mytilus chilensis*; cultured)



Time course of the intoxification - detoxification process

Changes in PSP toxin content in cultured mussels (*Mytilus chilensis*) were studied by mouse bioassay in 9 out of 15 sites between March 1st and June 1st, 2002.

- Average time between last non toxic and first toxic sample detection was 7.6 days (1 - 14 days)
- Average time between maximal PSP levels and 80 ug/100 g was 27.8 days (7 - 44 days)
- Average time between 80 ug/ 100 g to undetectable levels was 38.6 days (largest variability; 8 to 143 days). This was ca. 40% longer than period spent in high toxicity.

The time course revealed several characteristics observed in other species and locations (Bricelj & Shumway, 1998)

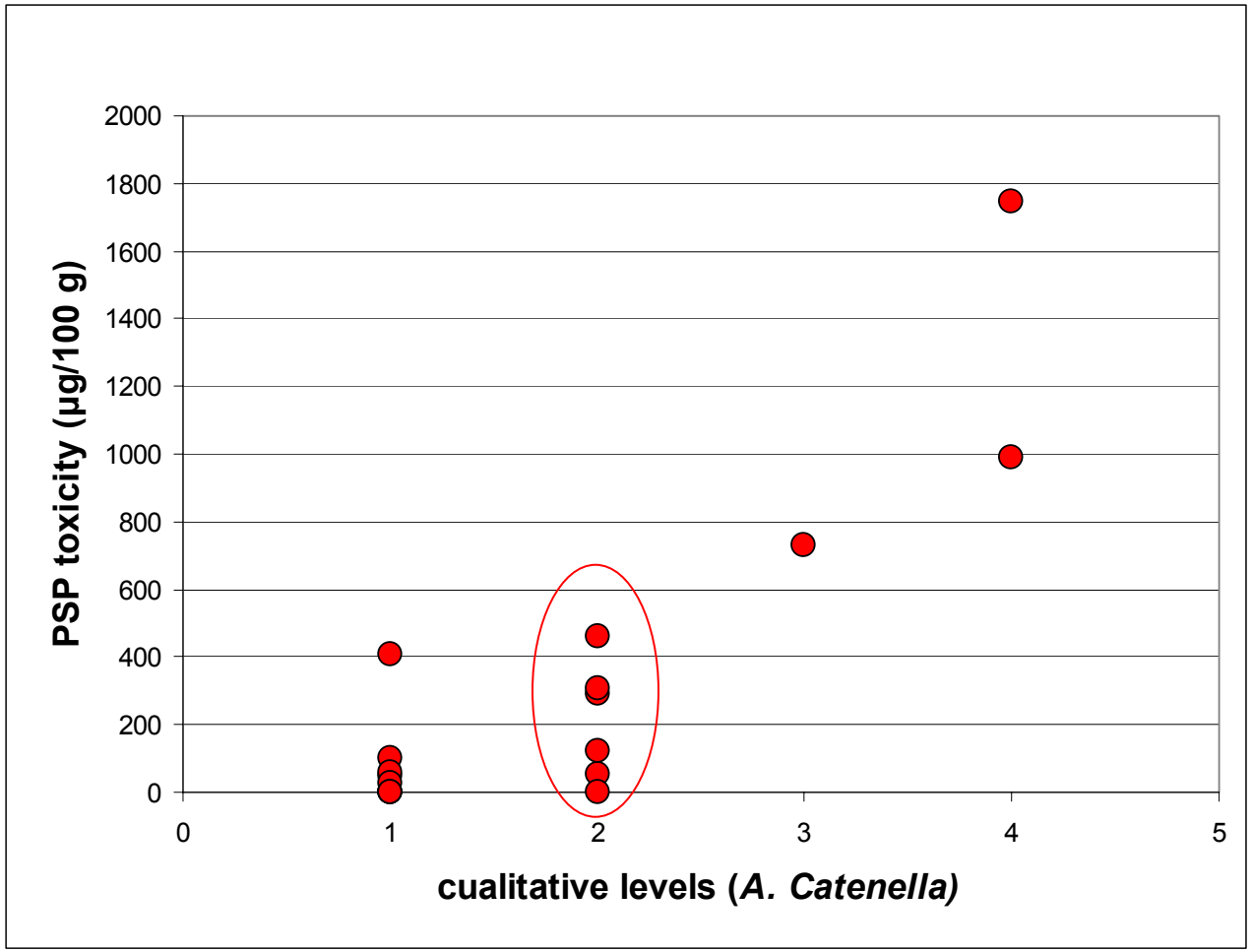
- In four sites with more complete time series, the detoxification phase could be approximately described as a bi-exponential process.

Fast rate: 3.82 days
Slow rate: 69.26 days

- Observed average rates and times of detoxification were similar to values found for fast detoxifiers (Bricelj & Shumway, 1998).

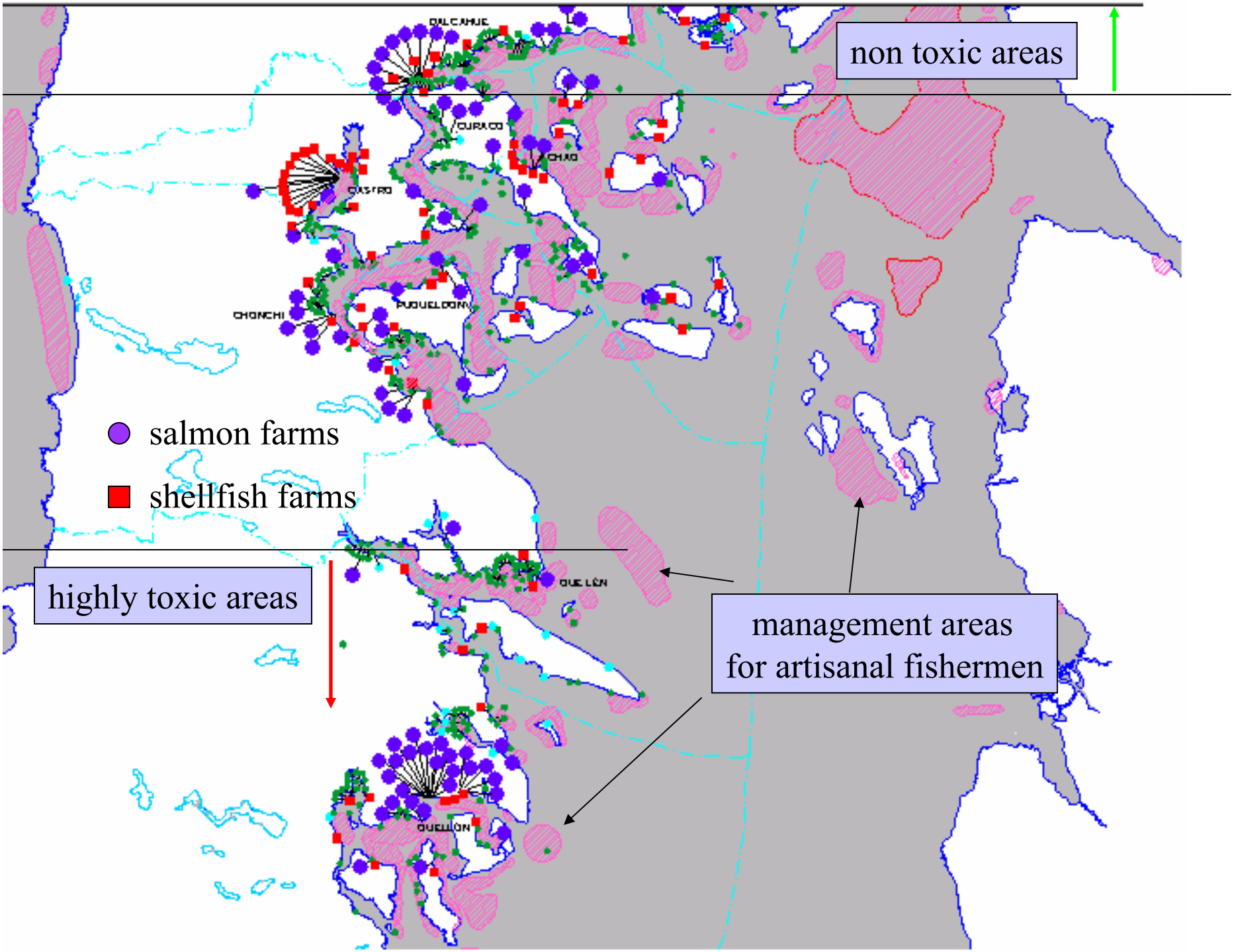
- The intox/ detox process was shown to be asymmetric with apparent faster rates of intoxication

Less than 2 *A. catenella* cells/mL produce up to 420 ug/100 g



Statement of the problem

- geographic complexity
- large number of sites
- over 200 aquaculture sites only in Chiloé
- distant natural shellfish beds
- large distances to laboratories
- unknown intra-population variability
- labor intensive industry


non toxic areas
salmon farms
shellfish farms
highly toxic areas
management areas
for artisanal fishermen
CHONCHI
QUELLÓN

Statement of the problem

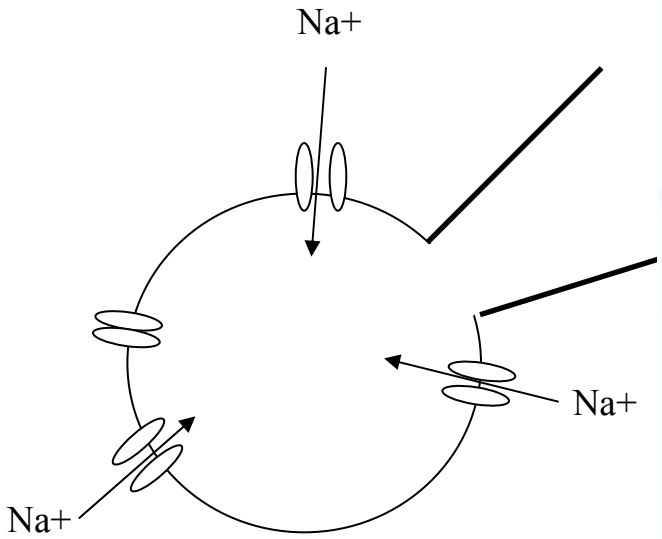
- geographic complexity
- large number of sites
- over 200 aquaculture sites only in Chiloé
- distant natural shellfish beds
- large distances to laboratories
- unknown intra-population variability
- labor intensive industry

- LIMITATIONS OF CURRENT REGULATORY METHODS

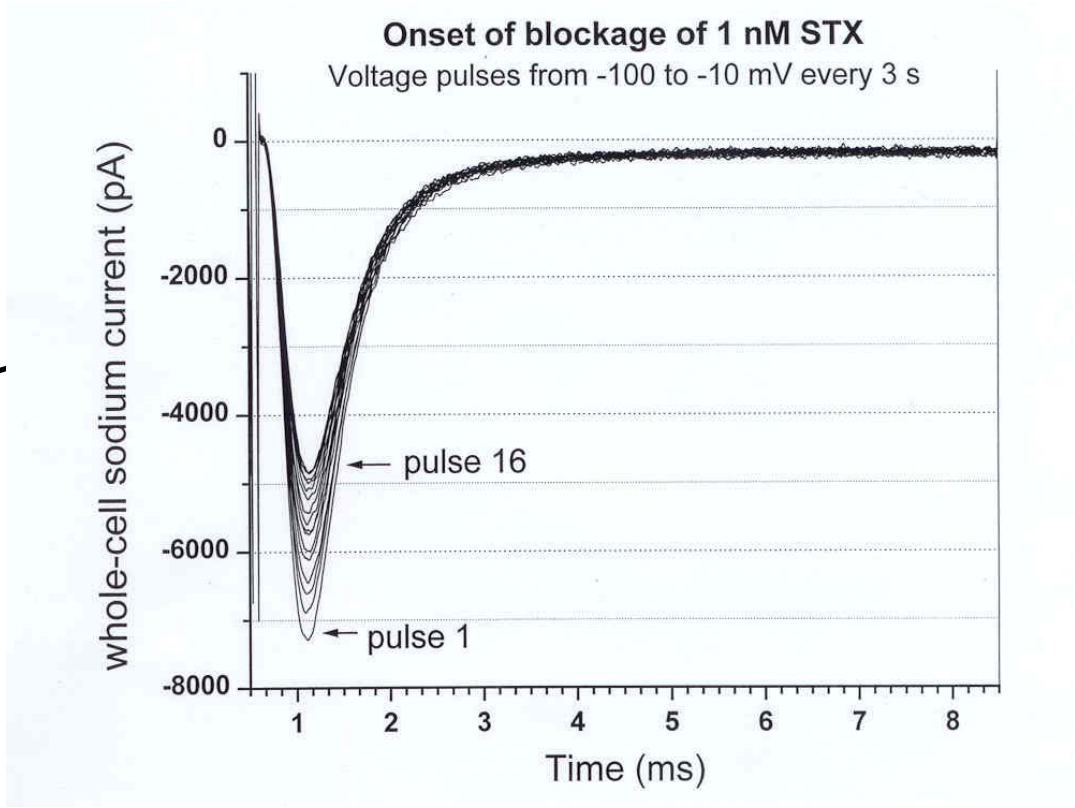
UNCERTAINTIES OF THE MOUSE BIOASSAY FOR PARALYTIC SHELLFISH POISON

COMPARISON WITH AN ELECTROPHYSIOLOGICAL ASSAY

The “Electrotest”

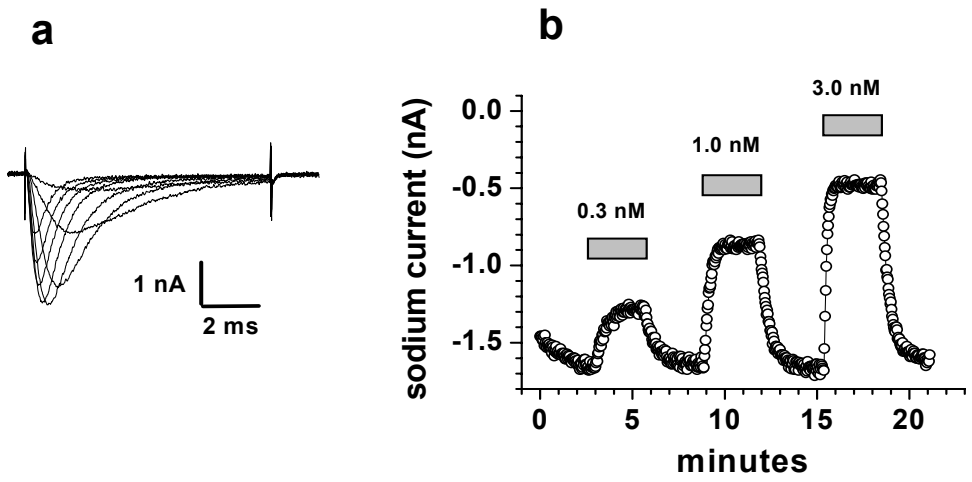


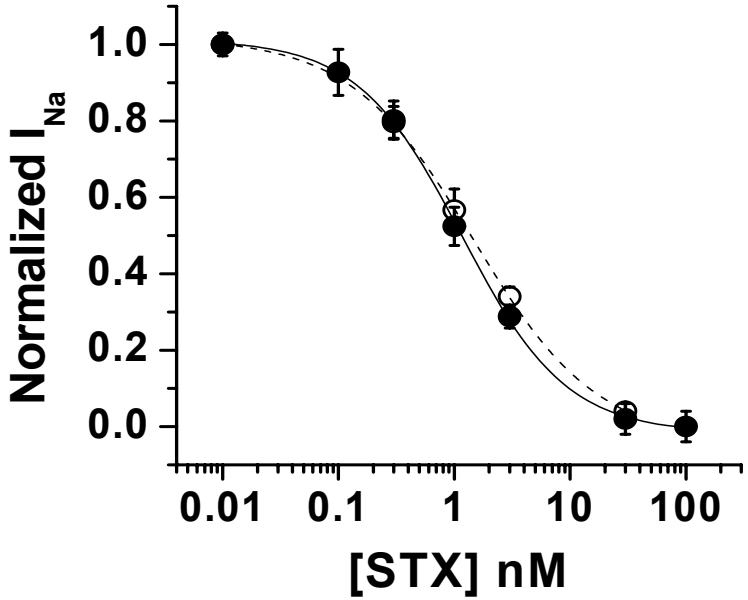
Patch clamp, whole-cell mode



HEK 286 cells stably transfected with
μ1 rat skeletal muscle Na channel isoform

Vélez et al., Toxicon, 39: 929-935 (2001)





(STX FDA)
IC_{50} = 1.46 ± 0.05 nM: n = 5
(STX Sigma)
IC_{50} = 1.17 ± 0.05 nM; n = 12

1 nM (=) 0.04 µg/100g
action level = 80 µg/100g

Electrotest is ca. 2000x more sensitive than the mouse bioassay

Vélez et al.,Toxicon, 39: 929-935 (2001).

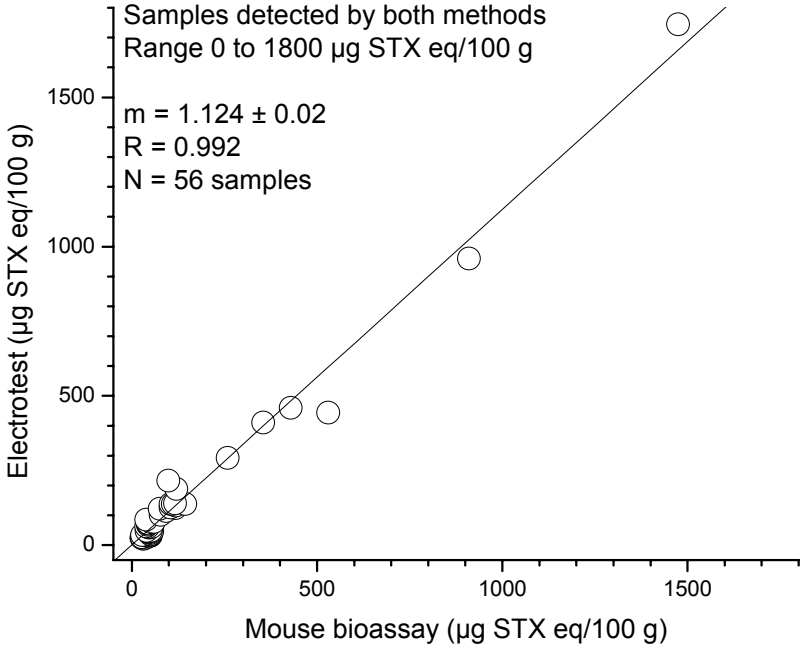
RESULTS

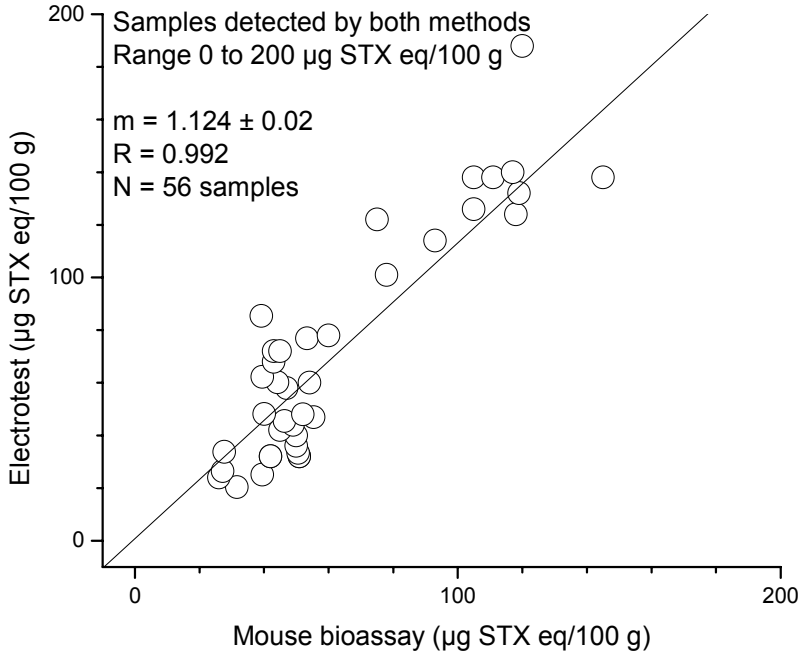
A set of 157 samples was analyzed by the electrophysiological assay and the AOAC mouse bioassay (MB).

Levels of toxicity determined by the electrophysiological assay ranged from 1.1 to 1744 µg STX eq/100 g.

All samples higher than 60 µg STX/100 g (N = 56) were detected by the mouse bioassay (MB) and a good correlation was found between the two methods.

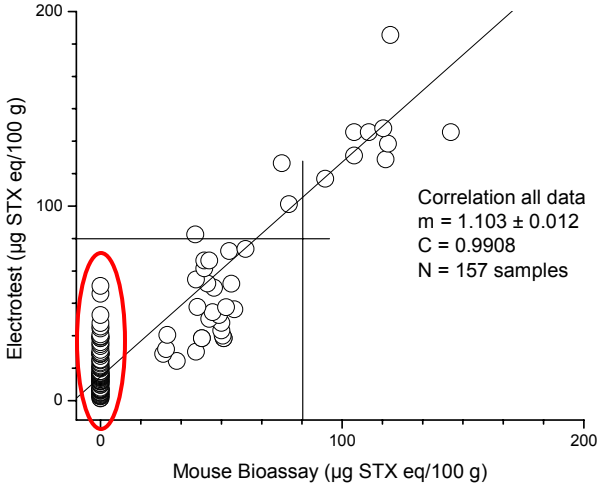
In contrast, only 20.3% of all samples under 60 µg STX/100 g (26 out of 128) could be found to contain PSP as measured by MB.

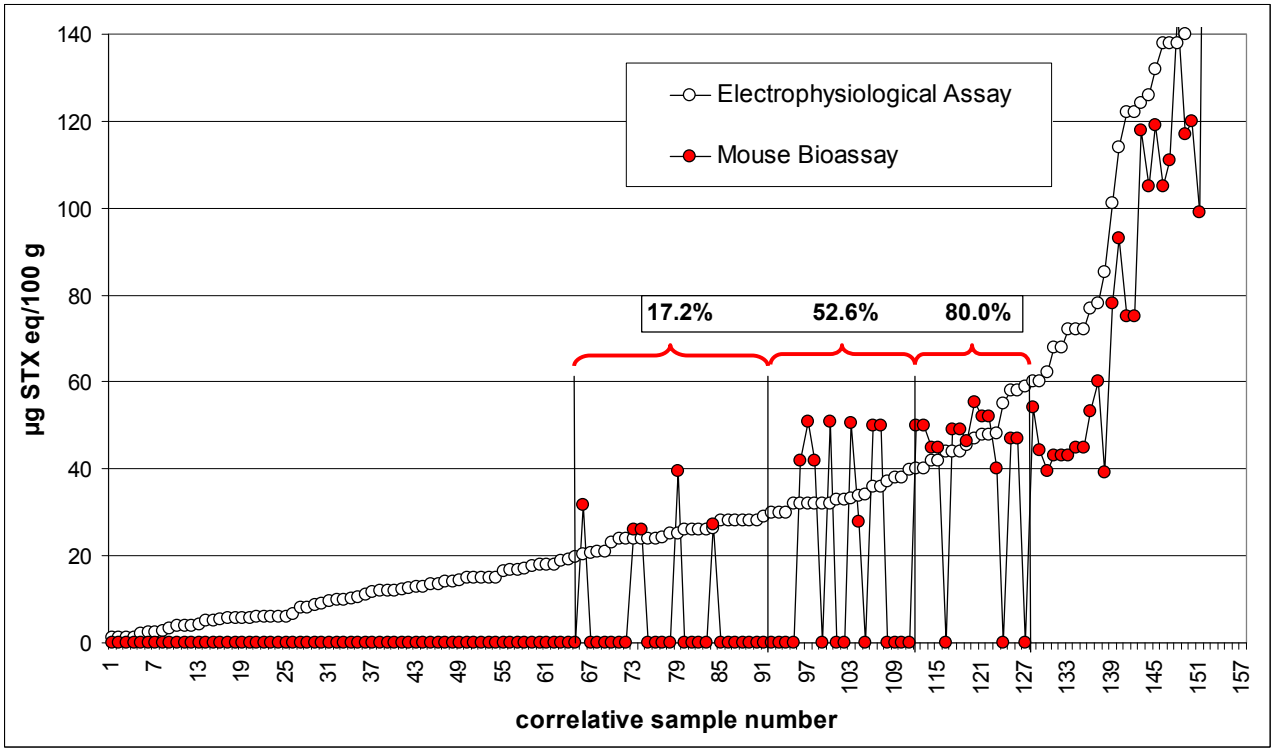


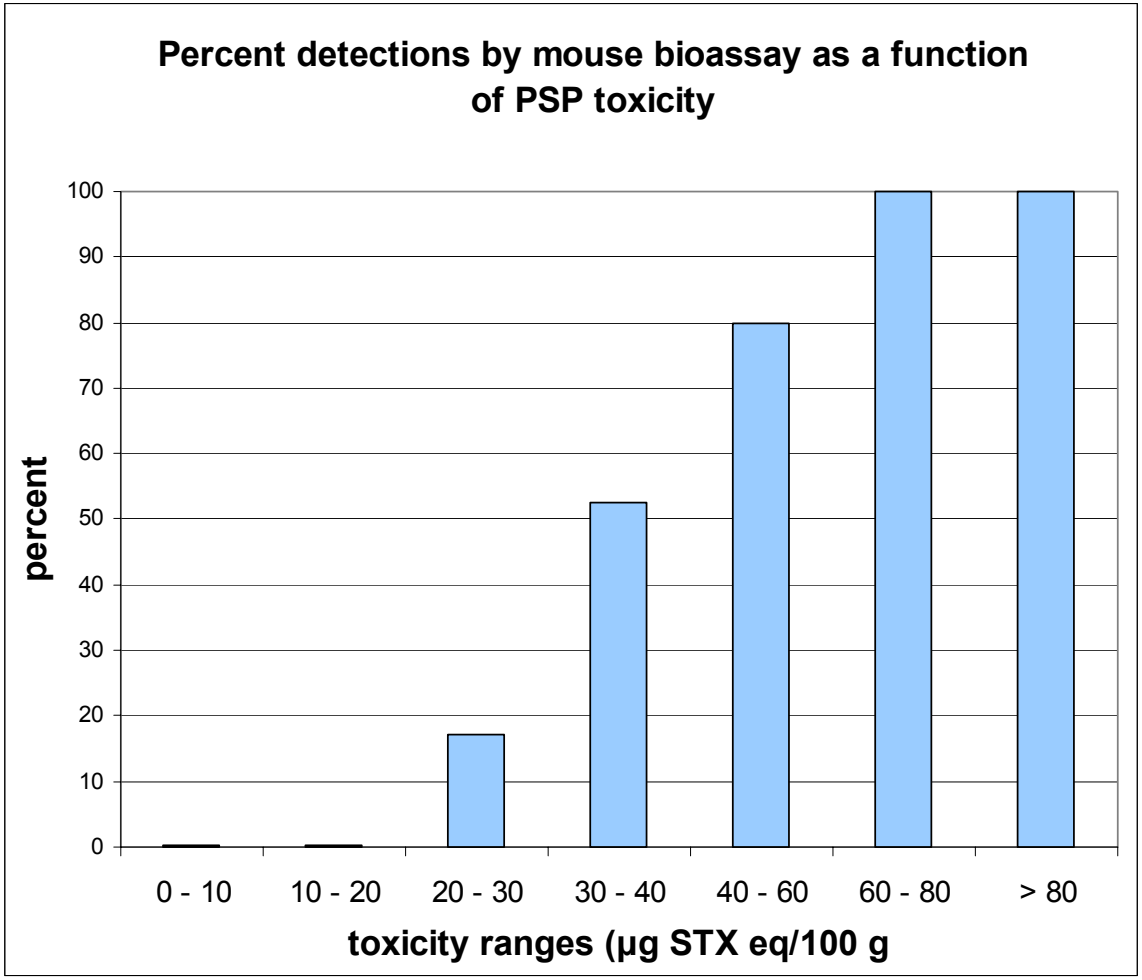


UNCERTAINTIES IN THE MOUSE BIOASSAY

Closer examination of lower toxicity ranges confirmed that management decisions taken solely on the basis of MB results are increasingly risky for samples below 60 µg STX/100 g.







TOXICITY RANGE (µg STX eq/100)	% CHANCE TO BE DETECTED
40 and 60	80.0
30 and 40	52.6
20 and 30	17.2

CONCLUSIONS

This is the first estimation of the operational detection limit of the AOAC mouse bioassay for PSP toxins obtained with an independent methodology that directly measures the funcional effect of saxitoxins.

Results confirm that the AOAC MB is a robust regulatory tool for samples that exceed 60 µg STX/100 g and suggest that an operational detection limit for the MB should be set at 40 µg STX eq/100 g.

Calculated values obtained from death times below 40 µg STX eq/100 g **are meaningless** and should be reported as a “below the detection limit” result.

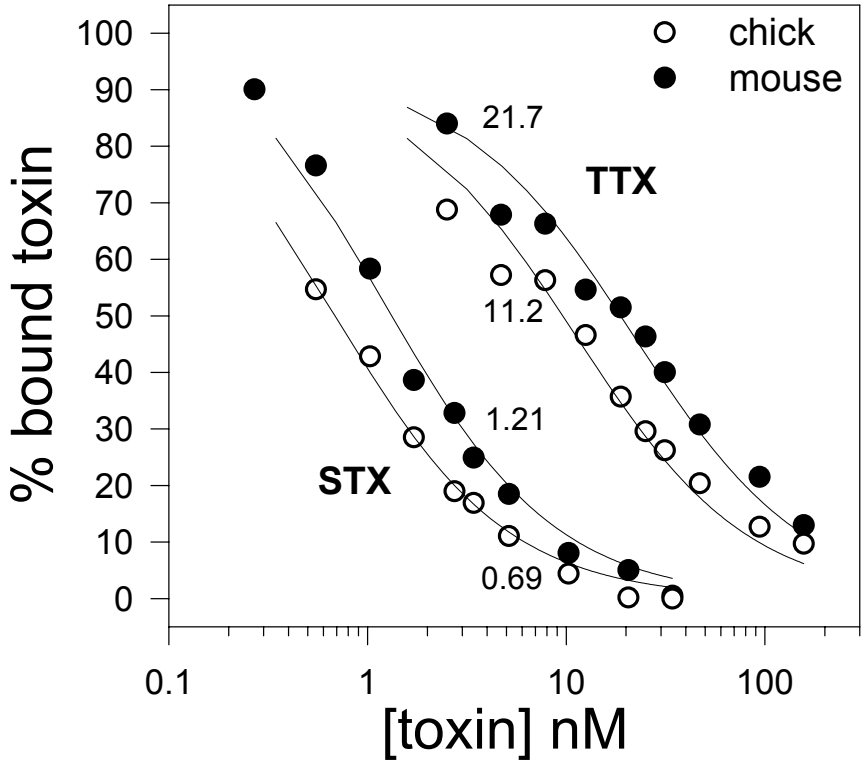
These observations confirm the utility of the patch clamp technique in PSP toxicity measurements and demonstrate the need to modify current monitoring strategies with the aim to determine the intra-population variability of PSP toxicity in an aquaculture site or shellfish bed.

UNCERTAINTIES OF THE MOUSE BIOASSAY FOR PARALYTIC SHELLFISH POISON

THE NEED FOR HIGH-THROUGHPUT RECEPTOR BINDING ASSAYS FOR MARINE TOXINS

PREVIOUS EXPERIENCE IN CHILE

[^{3}H]STX displacement curves

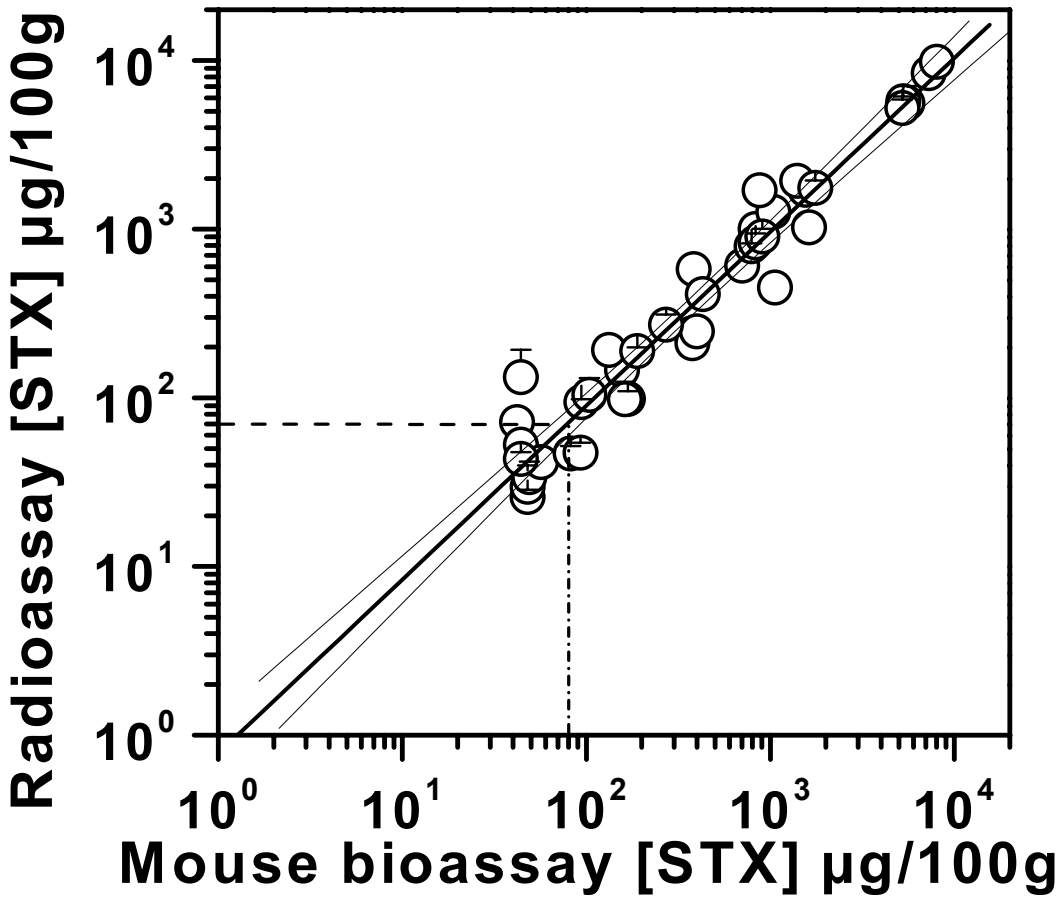


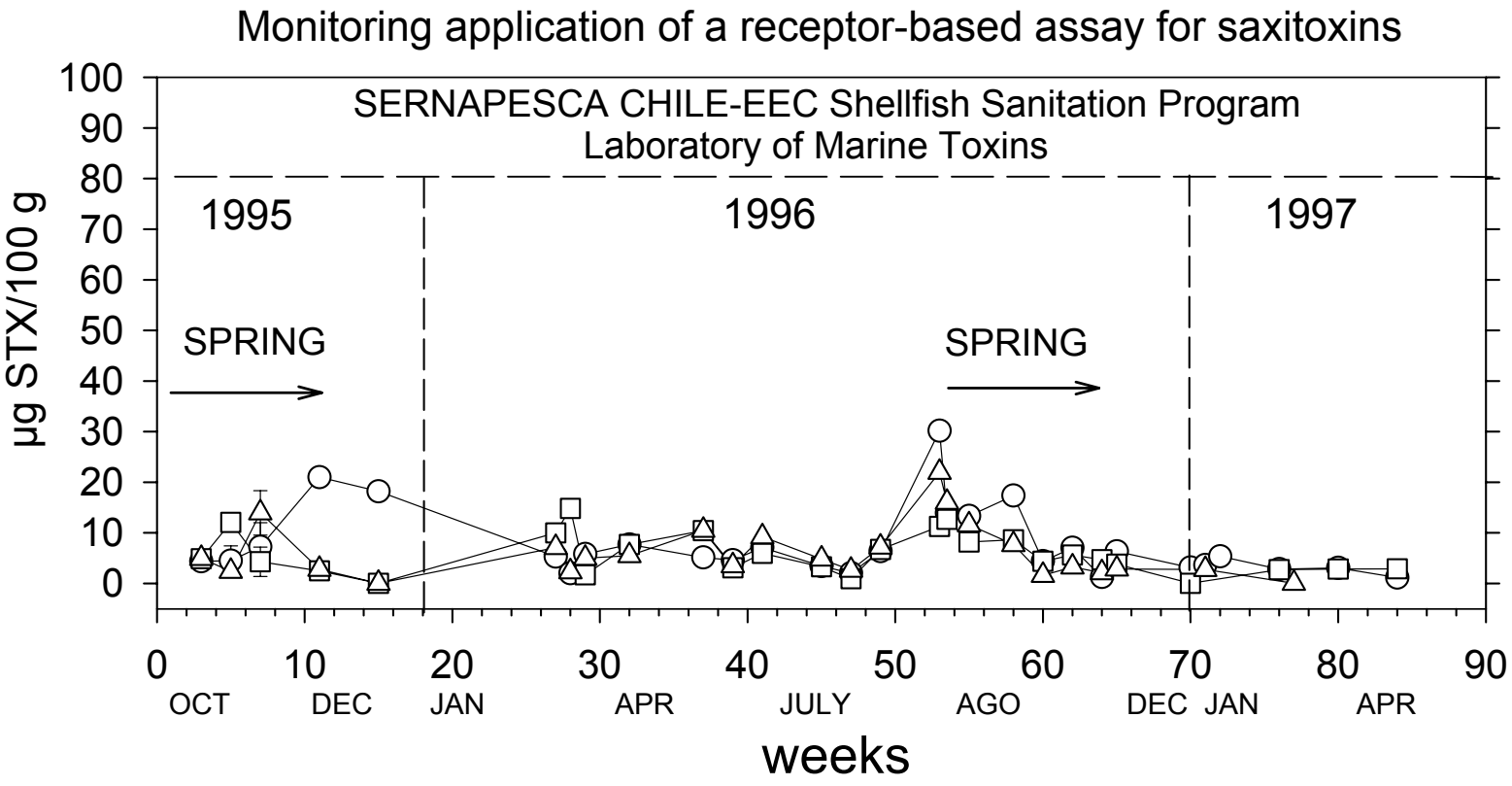
Parameters from Scatchard analysis

membranes	**K_D (nM)**	**B_{max} (pmol/mg)**
CF-1 mice	1.39±0.78	0.91±0.01(n=3)
chick	0.80±0.41	1.68±0.01(n=3)

Vélez, P., Sierralta, J., Alcayaga, C., Fonseca, M., Loyola, H., Johns, D.C., Tomaselli, G.F. and Marban, E. and **Suárez-Isla, B.A**. "A functional assay for paralytic shellfish toxins that uses recombinant sodium channels". Toxicon, 39: 929-935 (2001).

Correlation radioassay vs. bioassay
R=0.972; N=41 samples (1998)




Monitoring application of a receptor-based assay for saxitoxins
SERNAPESCA CHILE-EEC Shellfish Sanitation Program
Laboratory of Marine Toxins
1995
1996
1997
SPRING
SPRING
µg STX/100 g
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
OCT
DEC
JAN
APR
JULY
AGO
DEC
JAN
APR
weeks

Previous experience

RBA for saxitoxins was routinely applied from 1995 to 1997 to classify new aquaculture sites in the north of Chile

Commercially available tritiated STX was used

RBA results were confirmed by mouse bioassay

A manual version was used (filtering manifold and a conventional scintillation counter for tritium)

A trained operator could process between 20 and 40 samples per workshift

Sensitivity: 0.04 ug/100 g (bioassay 40 µg/100g)

Comparison bioassay vs. radioassay

Method	supplies	equipment	salaries	number of assays per workshift
Bioassay	mice reagents	Animal facility	one operator	10-15
Radioassay (manual version)	STX* reagents	filtering manifold access to scintillation counter	one operator	30-40
Radioensayo (high throughput)	STX* reagents plates	MICROBETA Wallac microplate counter	one operator	>200
Electro physiological assay	Cold STX no mice	set-up cell culture facility	one trained operator	10-15

Comparison bioassay - radioassay

Method	Sensitivity	Interferences	Variability
Bioassay	ca. 40 ug STX eq/100 g	Cd, Zn fatty acids	+/- 20%
REGULATORY LEVEL	80 ug STX eq/100 g		
Radioassay	0.04 ug STX eq/100 g provides early warning	minimized by high dilutions	+/-5,0%
Electro-physiological assay	0.04 ug STX eq/100 g provides early warning	minimized by high dilutions	+/-8,3%

CONSEQUENCES FOR MANAGEMENT

a. After toxic phytoplancton detection (T1) increase sampling frequency	Delay beginning of site closure
b. Decrease frequency during period above 80 ug/100 g	Lower costs
c. Increase sampling frecuency below 80 ug/100 g	Advance reopening of site

END RESULT

Diminish closure days

Current issues and the future

The first PSP outbreak in the area with the highest productivity generated:

The first CONICYT Red Tide Research Program (5 grants allocated, 1 million U$)
(Program has 5 years duration)

Current grant themes

- dinoflagellate genomics
- oceanography and modelling
- monitoring techniques
- early warning methodologies, expert systems and education LABTOX grant
- organism detection

Research groups will coordinate their studies. Program will continue

A new Acquaculture Research Program (6 grants, 2 million U$)

- species substitution
- shellfish detoxification LABTOX grant

IAEA-CCHEN-UCHILE TC Projects CHI/07/009 and CHI/07/010
"Application of Receptor Binding Assays for Mapping and Monitoring of Shellfish Poisoning Toxins".

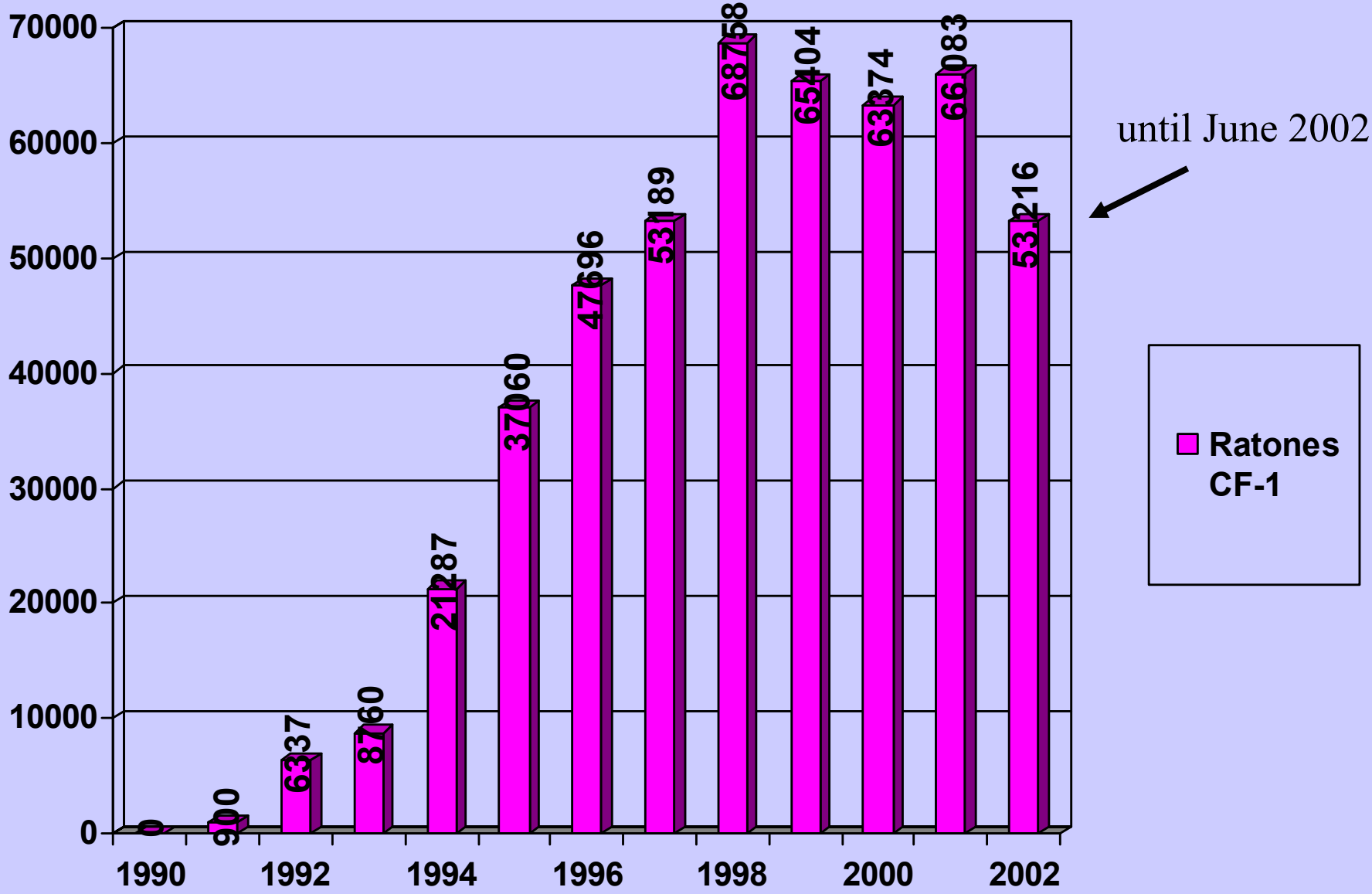
General Objective. The general objective of this project is to develop and transfer robust application formats of a receptor binding assay (RBA) for the detection of Paralytic Shellfish Toxins (saxitoxins) in shellfish and water samples, to be performed in remote laboratories of regional health services and hospitals, and shellfish processor plants.

This new technology will increase the number of analysis per work shift, decrease the response time, provide early warning information to regulators and producers and significantly reduce costs per analysis. To this end local synthesis of radiolabeled saxitoxin, training workshops, technology transfer activities, intercalibration exercises and improvements in analytical reference technologies and functional assays will be implemented.


NO EXTRAER
MARISCOS
MAREA
ROJA

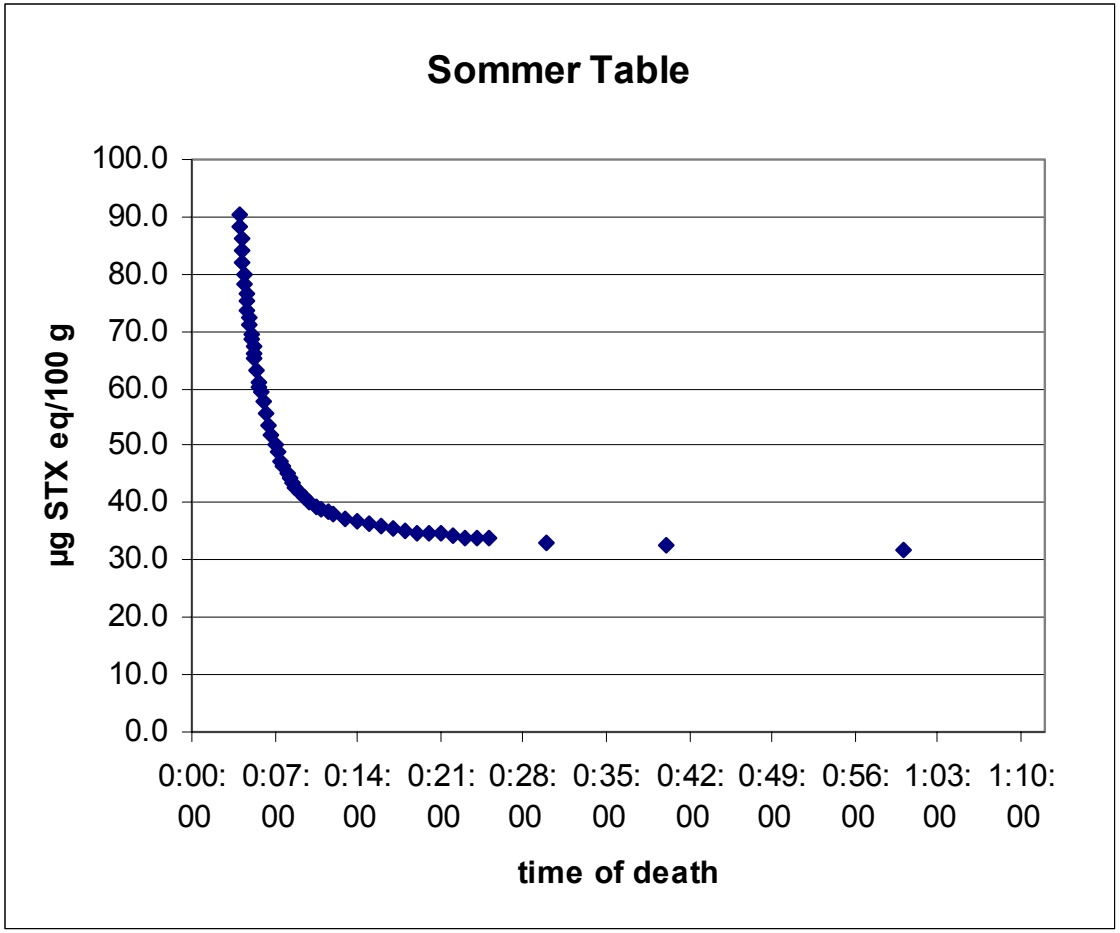

FDA site visit
July 2002

CF-1 mice produced by the Institute of Public Health (1990-2002)

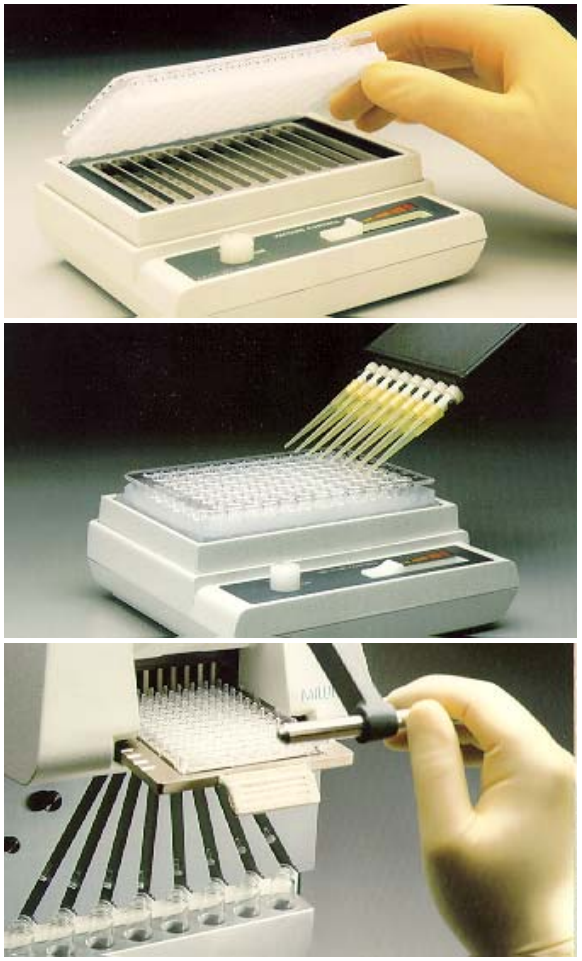


Convert median of death times to “mouse units” (MU) after Sommer Table, correct for mouse weight (CMU)

µg STX eq./ 100 g = CMU x CF x Dilution x 200



Receptor binding assay (RBA) in high throughput format



Perkin Elmer WALLAC MICROBETA counter