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Acethylcholinesterase : Methodology development of a biomarker and challenges of its application for biomonitoring.

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Abstract

This study presents a model of classification with the ACHE biomarker to assess the neurological state of mussels in Atlantic area after the Erika oil spill. Acetycholinesterase (AChE) activity is recognized as a well established environmental biomarkers of neurotoxicity and its track record for biomonitoring could be very instructive for the future implementation of the structured monitoring programme CEMP (Coordinated environmental monitoring programme). AChE (EC 3.1.1.7) is present in most animals to assess the early biological effects to neurotoxic contaminants in aquatic environments.
The earliest study along the French Mediterranean and Atlantic coasts was carried out at the end of 1980s. The presence of AChE has been demonstrated in a variety of marine organisms and specific field studies were done in fish, crustacean and mollusks. The French experience acquired in various national and european field studies and our implementation in the ICES working group of biological effects of the chemical contaminants since twenty years, lead us to recommend this biomarker (AChE activity) as a pertinent biomarker for future implementations into the CEMP. Indeed, the background response level (36.2 ± 6.2 $nmol.min^{-1}.mg\ protein^{-1}$) and assessment thresholds of AChE activity have been determined in mussel *Mytilus edulis* from a three year survey, after the Erika oil spill. This approach is very promising for the integrated monitoring CEMP and the implementation process of monitoring for the Marine strategy framework directive (MSFD).

Keywords: Acethylcholinesterase, biomarker, mussel, biomonitoring, classification of the ecological status, Coordinated Environmental Monitoring Programme, Marine Strategy Framework directive, Oil spill.

Introduction

The analysis of acetylcholinesterase (AChE; EC 3.1.1.7) activity in marine organisms has been shown to be a highly suitable method for assessing exposure to neurotoxic contaminants in aquatic environments. AChE is involved in of the rapid elimination of the acethylcholine (ACh) into choline and acetate, the major neuromediator of neuro-muscular activity. This probably contributed to the sustained interest of using this biomarker in environmental field investigations over the years. The ecological or physiological relevance of this biomarker resides in its involvement in many important processes for survival (neuro-muscular alertness that underlies avoidance and attack reactions for example) and reproduction (Corsi et al., 2007). AChE is an enzyme present in membranes located in the synaptic space at the neuro-muscular jonction The enzyme degrades acethylcholine in the synaptic space and reduce muscular stimulation. Inhibition of this enzyme leads to increased and sustained stimulation acetylcholine stimulation of muscle leading to muscle hyperstimulation, tremors and paralysis.
AChE is recognized to be one of the oldest environnemental biomarkers (Payne et al., 1996). It was initially used as biomarker of exposure to organochloride pesticides to assess the neurological integrity of exposed organisms. AChE activity method is applicable to a wide range of species and has the advantage of detecting and quantifying exposure to neurotoxic substances without a detailed knowledge of the contaminants present. Being an indicator of neurotoxic effects, AChE has traditionally been used as a specific biomarker of exposure to organophosphate and carbamate pesticides (e.g. Coppage and Braidech, 1976; Day and Scott, 1990; Bocquené and Galgani, 1998; Printes and Callaghan, 2004; Hoguet and Key, 2007). More recently, other groups of chemicals present in the marine environment including heavy metals, detergents and hydrocarbons were shown to affect its activity (Zinkl et al., 1991; Forget et al., 2003, Brown et al., 2004). Payne et al., 1996 suggested that this old biomarker may have a new future in biological monitoring and assessment programme. After fourtheens years, its track record for biomonitoring could be very instructive for the future implementation of the structured monitoring programme CEMP (Coordinated environmental monitoring programme). As some other countries in Europe, the application of AChE was initiated in France since the end of the eighties (Bocquené et al, 1990). AChE was studied in different species along the Atlantic and the Mediterranean French coasts (Galgani et al 1992; Bocquené et al., 1993; Burgeot et al., 1996,). Seasonal variations were studied in each species according to their specific habitats, age and sex (Minier et al., 2000, Bodin et al., 2004, Belieaff and Bocquené, 2004, Cailleaud et al., 2009). It was well established that the increase of water temperature significantly affects the expression of the AChE activity. Indeed, temperature can change the activity of the enzymes by changing the protein conformation and the catalytic efficiency or binding capacity. A more pronounced effects of salinity over temperature could also affect the AChE expression in copepod species (Cailleaud et al., 2007) The methodology for analysis and the assessment criteria are now largely documented for the integration of biomarkers in the CEMP (OSPAR, 2007) in 2000. Background levels of imposex and assessment criteria were determined for the lysosomal membrane stability in mussel (MSFD GES Task group 8, 2010) but few experiments were done with enzymatic biomarkers of exposure. The ICES working group on biological effects of chemical contaminants (WGBEC) recommended AChE for biomonitoring application in either fish and shellfish and its inclusion into the CEMP (ICES 2008). The methodology for AChE analysis is well developed and available through ICES TIMES (Bocquené and Galgani, 1998) and background response levels are proposed for different species. Because of lack of assessment thresholds and established quality assurance in the Biological effects quality assurance in

monitoring programmes (www.Bequalm.org), AChE is not yet include in the Joint Assessment Monitoring Programme guidelines (Thain et al., 2008).

The objective of this study was to determine a AChE activitie background response level and assessment thresholds in mussels *Mytilus edulis* and along the French Atlantic coasts. One AChE data set acquired during three years (2000 to 2002) after the oil spill of the Erika in December, 1999 (Bocquené et al., 2004), was used in order to propose a model of AChE classification in different impacted area situated around the Loire estuary.
The main purpose of this approach was to:

- Define a background response level and assessment thresholds during the restoration period (2001-2002) after the oil spill.
- Compare the assessment thresholds for AChE during the acute exposure in 2000, just after the Erika oil spill.

Methods

Temporal and spatial survey
A 3-year survey was perform to follow changes of many biological markers in mussels (Mytilus edulis) exposed *in situ* to the oil that came ashore after the wreck of the Erika tanker on the Brittany (France) coast in December 1999 (Bocquené et al., 2004). In this study, we used the AChE enzymatic activities data from January 2000 to December 2002. The study encompassed both the acute contamination in 2000 and the restoration phase returning to a state of equilibrium in 2001 and 2002. The period from 2001 to 2002 was thus selected for the determination of the background response level and the assessment thresholds for the development of a model of the classification with the AChE enzymatic biomarker. Six stations (Pen Bé, Pointe de Castelli, Le Croisic, Pointe de Chemoulin, Maison Blanche, Tresson) were compared (Fig 1) and the AChE model of classification was then applied during the period of acute exposure in 2000. 30 mussels were collected at each sites. Gills were immediately removed and pools samples from 5 individual were stored in liquid nitrogen before the AChE enzymatic activities measurements. AChE enzymatic activities were analyzed according to the methode described by Bocquené et al., 2004.

The temperatures (°C) of the surface waters were measured with one *in situ* sensor in le Croisic station (Fig 1) from January 2000 to December 2002 (database of the national network Ifremer/Quadrige/Rephy)

Statistical analyses
The AChE activity data was modeled by a Gaussian linear regression model against temperature (°C), based on the observations of the years 2001 and 2002. AChE was significantly explained by the Temperature ($p < 0.001$).
The following linear regression equation was obtain:
AChE activity = 29.79630 + (0.47 x Temperature)

The model residuals, which measure departure from the model fit (estimated Background response level) are clustered in three groups using the k-means method (Hartigan et al., 1979). The data given by x is clustered by the k-means method, which aims to partition the points into k groups such that the sum of squares from points to the assigned cluster centers is minimized: The classification of a give AChE value is one of the three groups (Classes) depends on the difference between the analyzed value of AChE and the AChE value adjusted by the model:

Residues = AChE – (29.796 + 0.474 * Temperature)

Group (Classes)	Residues
Red	< - 4.016
Yellow	[-4.016 , 4.939]
Green	> 4.939

According to the WFD CIS 2005 guidance and the MSFD GES task Group 8 (2010) recommendations, three color traffic light scheme (Red : bad ; orange : moderate; green : good) has been selected to represent the three group of effects.

Results

Background response level of AChE

The background response level of AChE was determined from the monthly enzymatic measurements in the gills of mussels from January, 2001 to December, 2002 (Fig 2). This period of return towards a state of equilibrium of the ecosystem was selected to determine the background response levels of AChE in the vicinity of the Loire estuary (Fig 1). The background response levels corresponds to a mean value of the AChE during a period of chronic exposure. The acute period of exposure in 2000 was not selected. Weekly temperature measurements were used to normalize the background response level (Tab 1). The seasonal variations of temperatures are distributed from 7°C to 21°C during 2001 and 2002. The amplitude of the temperature uncorrected AChE background response level ranged from min (33.4 ± 3.4 nmol.min^{-1}.mg protein^{-1}) and max (39.2 ± 8.5 nmol.min^{-1}.mg protein^{-1}) with a mean of 36.2 ± 6.2 nmol.min^{-1}.mg protein^{-1}. The background response level describes a seasonal variations (Fig 2) with highest values in summer.Two important periods of variations appears with lower values from November to March and higher values from April to October. The period between March and April is a critical period during the physiological cycle of the mussel because of the gametogenesis activation (Bayne, 1976).

Assessment threshold: AChE model of classification

Assessment thresholds were determined during the aftermath of the Erika spills after cleaning in 2001-2002 (Fig 3). This period can be considered to a chronic period less impacted by the oil spill. The objective was to build a model of classification of neurological states of mussels according to the recommended ICES study group (SIMGC : Study group integrated monitoring of contaminants and biological effects) with a division of responses between three classes (ICES 2010). The three classes would be categorized with a color code into “background” (green), “exposed” (yellow) and possibly deleteriously affected (red).
The AChE variations analyzed into six stations during 2001 and 2002 were integrated and represented in an annual seasonal cycle (Fig 3). Three classes of effects are determined during one years.
During the chronic period (the less impacted period from 2001 to 2002), the classification model identified different impacted stations (Fig 4). The calculation of the frequencies and percentages of the AChE activities in the three classes (Tab 2) allowed to compare different

impacts of the oil spill between the six sampled stations (Fig 5). The station Pointe de Chemoulin is the most impacted and the following classification is obtained : Pointe de Chemoulin> Tresson> Pen Bé>Le Croisic> Maison Blanche> Pointe de Castelli.
The profile of the AChE responses is very different between 2001 and 2002. Three years after the wreck of Erika and during the less impacted period in 2002, the AChE activities are only classified in a yellow or a red category. The highest variations of AChE activities observed in 2001 are mainly responsible of the three classes determined with the model of classification. 2001 is typically a period of a restoration and the three classes could illustrates a greatest effort of regulation characterized with highest AChE variations.
During the acute period of exposure in 2000, the classification identified a most sensitive period with a greater impact of the oil spill. The highest frequencies and percentages (Fig 4) were calculated into the red classes (Tab 3). A graphical representation of the three color classes (Fig 6) easily compared the six sampled stations in the Loire estuary. The following classification is obtained : Pointe de Castelli > Pointe de Chemoulin> Maison Blanche >Tresson> Pen Bé>Le Croisic.
The spatial impact of the oil spill was different between the acute contamination in 2000 and the period of restoration in 2001-2002.

Discussion

A quantitative assessment thresholds are needed for effects data to be included in environmental assessment programmes (Sandström et al., 2005). Background levels of imposex in gastropod and assessment criteria were determined with the lysosomal membrane stability in mussel (MSFD GES Task group 8, 2010) but few experiments were done with enzymatic biomarkers of exposure. The period seems now particularly suited to determine assessment criteria for AChE in mussel and to evaluate it the potential of integration in the CEMP and in the MSFD.

Establishment of background response level :

The potential of application of biomarkers in biomonitoring was demonstrated on many studies about twenty years ago. A list of biomarkers of interest was recommended by the ICES (ICES , 2008) for an integration in the CEMP. Among the assessment criteria, the definition of the background response level is mandatory (OSPAR, 2007). The background response level, can be defined from an average of the enzymatic activities AChE obtained by species and by specific habitat during several years. It is important to know the natural limits of variability in AChE activity in the species of interest to assess the significance of the observed depression in activity. Furthermore the physiological consequence of deviations from these background levels should also be understood. For example, a 40 % decrease in AChE activity was associated to decrease of feeding in *Gammarus fossarum* exposed to methomyl concentrations (Xuereb et al., 2009). The decreased on feeding rate was also associated to increased lysosomal membrane stability. In another study with *Mytilus galloprovincialis*, mussels exposed to propanolol and acetomiphen, the feeding rate was significantly lower in the propanolol group and increased in the acetaminophen treatment group (Solé et al., 2010). AChE activity was significantly reduced by both drugs. These studies show the ecological significance of the AChE activity measurement at the behavioural (feeding) level which constitutes a critical parameter for survival.
A series of data obtained during the two years survey following the wreck of the Erika allowed to determine the background response level of the AChE in mussels and to evaluate

the neurosuppressive effects of oil spillage of mussels in the Loire estuary. The background response level follows the temperature changes which is integrated in the AChE measurements and the annual physiological variation of the mussels.

AChE Model of classification

A model classification based on a robust AChE activity model for wild mussel populations is proposed. The temperature appeared as a major factor for the explanation of the model. The mussel AChE activities analysed by Bocquené et al;, (2004), showed clear seasonal variations every years of the survey with highest activities during the summer months. After the oil spill, a higher PAH concentrations were also identified in mussels tissues in autumn and winter (Tronczynski et al., 2004). The seasonal variations of the PAHs were attributed to lower metabolizing capacities because of lower temperature and higher lipid contents before the spawning period.

In *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea, mean values of AChE values vary two-fold depending on season, following closely changes in temperature (Leiniö and Lehtonen, 2005). The metabolism of the bivalves and more specifically the initiation of important physiological changes such as gametogenesis or the spawning are never provoked by one single stimulus but only when an appropriate combination of stimuli occurs (His et al., 1999). The gonad development in *Mytilus edulis* is controlled by internal neuro-endocrine factors and the external factors such as temperature and food act as synchronizers (Thompson et al 1979). AChE could be also involved in the control of many essential physiological functions, such as frontal ciliary activity of gill epithelium, temperature resistance, ciliary activity for transport of suspended particulate, valve opening and embryo development (Corsi et al., 2007) . Because of the combined effects of different physico-chemical and physiological factors, the model of classification developed in mussels must be evolutive and should integrate more other factors (i.e salinity, gonad maturation and food quality).

The AChE model of classification was applied in 2000 during the period of the acute contamination, after the accident of the Erika (in December, 1999). Significant lower levels of AChE activities were mesured by Bocquené et al., (2004) during 2000 compared to 2001 and 2002, with a decrease of up 30% , at levels susceptible to induce significant changes at the behavioural levels namely at the feeding rate level. The comparison of the six exposed stations demonstrated that all the stations were greatly impacted in 2000 (Fig 4 and Fig 6) with a different impact beteween the same stations sampled in 2000 and in 2001-2002. (Fig 5 and Fig 6). The classification of the stations is completely different between the acute period and the period of restoration.

It seems possible to establish a model of classification with three classes of response for a spatial and temporal evaluation of the neurotoxic effects. Nevertheless, the interpretation of the ecological signification is always a challenge. During the chronic period in 2002 (Fig 4), the variability of AChE is lower than in 2000 and a classification following two classes (red and yellow) demonstrated a significant effect. But the high variability observed in 2000 and 2001 (Fig 4) could be explained by an elevated physiological effort of regulation during the acute contamination (Burgeot et al., 2006).

During the period of the restoration in 2001, the persistence of the PAHs contamination was demonstrated in a very located areas (Tronczynski et al., 2004). The higher mobilization of the fuel was observed especially after high tides and major storm during autumn and winter in 2001 and in winter in 2002. The deposited fuel was probably re-introduced into the water. The re-mobilization of the fuel from the oiled sediment on a located areas may generate high concentrations in surrounding organisms over a long period of time. The re-mobilization of the fuel was more elevated in 2001 than in 2002 and it could explain the greatest variability of AChE response in 2001.

Conclusion

The integration of biomarkers in a monitoring programme requires the validation of the assessment criteria and a well established quality assurance procedure. The development of assessment criteria was undertaken by the ICES working group of the biological effects of the chemical contaminants and with the contribution of working groups of experts in the marine monitoring (WKIMON: Working on Integrated Monitoring contaminants and effects in coastal and Open-sea areas and SGIMG). A background document was drafted for a list of biomarkers making consensus but more efforts will be required to attain integration in the JAMP and in the CEMP. The assessment thresholds was developed with the enzymatic biomarker AChE, from a series of data obtained after the Erika oil spill along the French Atlantic coast. Three classes (Red: effect, yellow: exposure and green: background) allow to identify a different spatio-temporal impact during the acute exposure and the period of the restoration. Because of the combined effects of different physico-chemical and physiological factors, the model of classification must be evolutive and should integrate more other factors in the future. This model could be also applied with some other enzymatic biomarkers and integrated in a multi-biomarker approach. This model approach developed with an enzymatic biomarker of exposure in mussel is very promising for future monitoring initiative in the CEMP and in the MSFD.

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Figures and legends

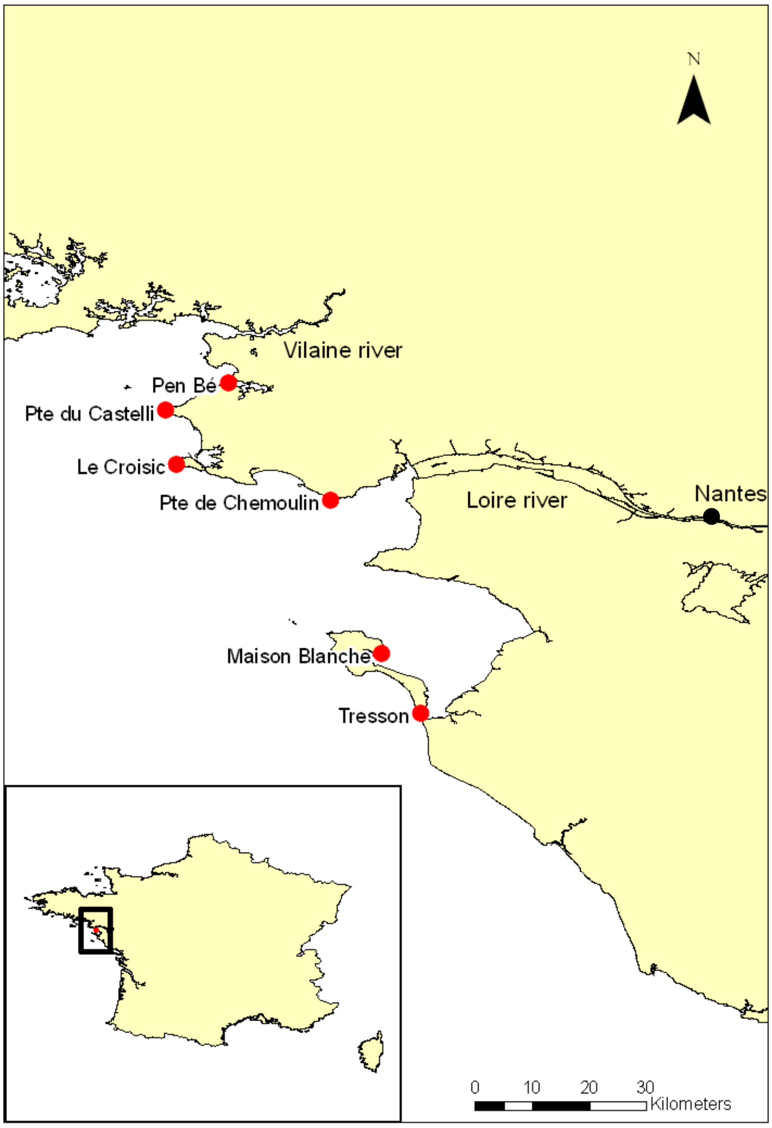

Figure 1 : Sampling stations for the collection of mussels (*Mytilus edulis)* during three years (2000-2002) after the Erika oil spill along the French Atlantic coasts, in the Bay of Biscay.

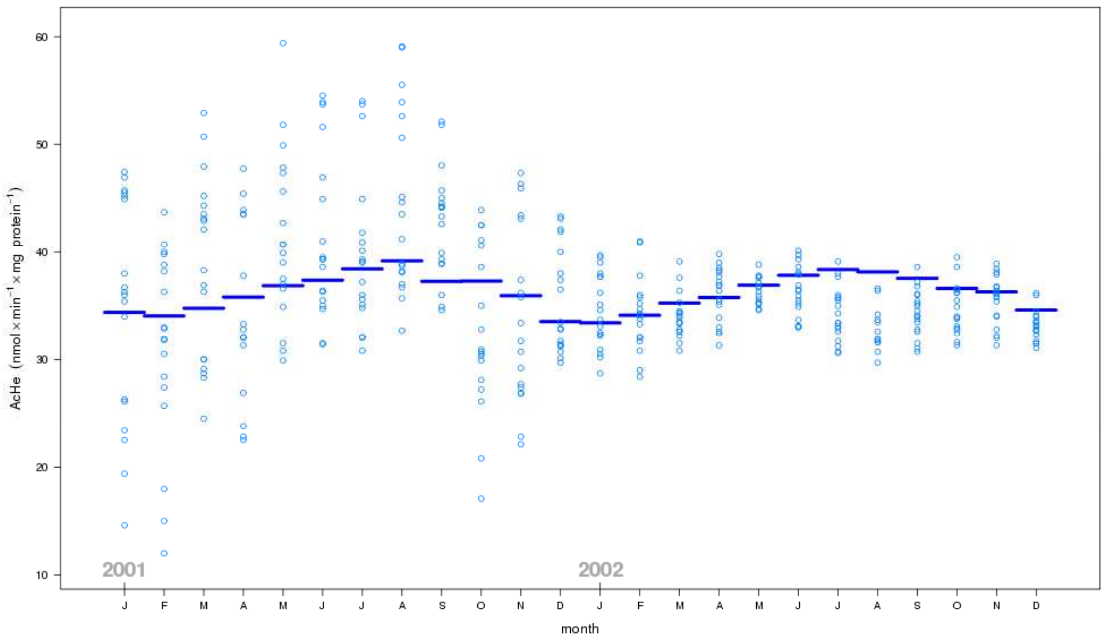

Figure 2: Background response level of AChE activities analyzed in mussel *Mytilus edulis* and sampled into the six stations from January, 2001 to December, 2002. The background response level (- - - -) include the temperature fluctuations during the period of restoration.

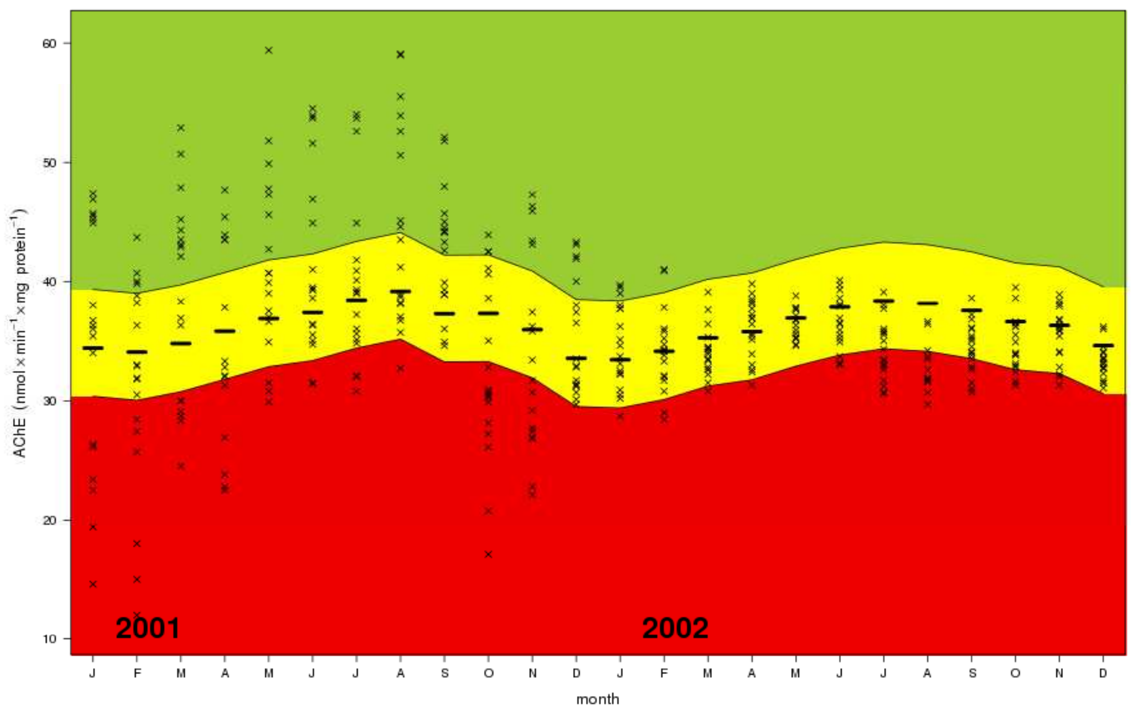

Figure 3 : Model of classification determined with three classes of neurotoxic effects during the period of restoration (chronic period) in 2001-2002. The background response level (- - - -) include the temperature fluctuations. A color code is affected to every points distributed following the three classes (Red : bad ; orange : moderate and green: good).

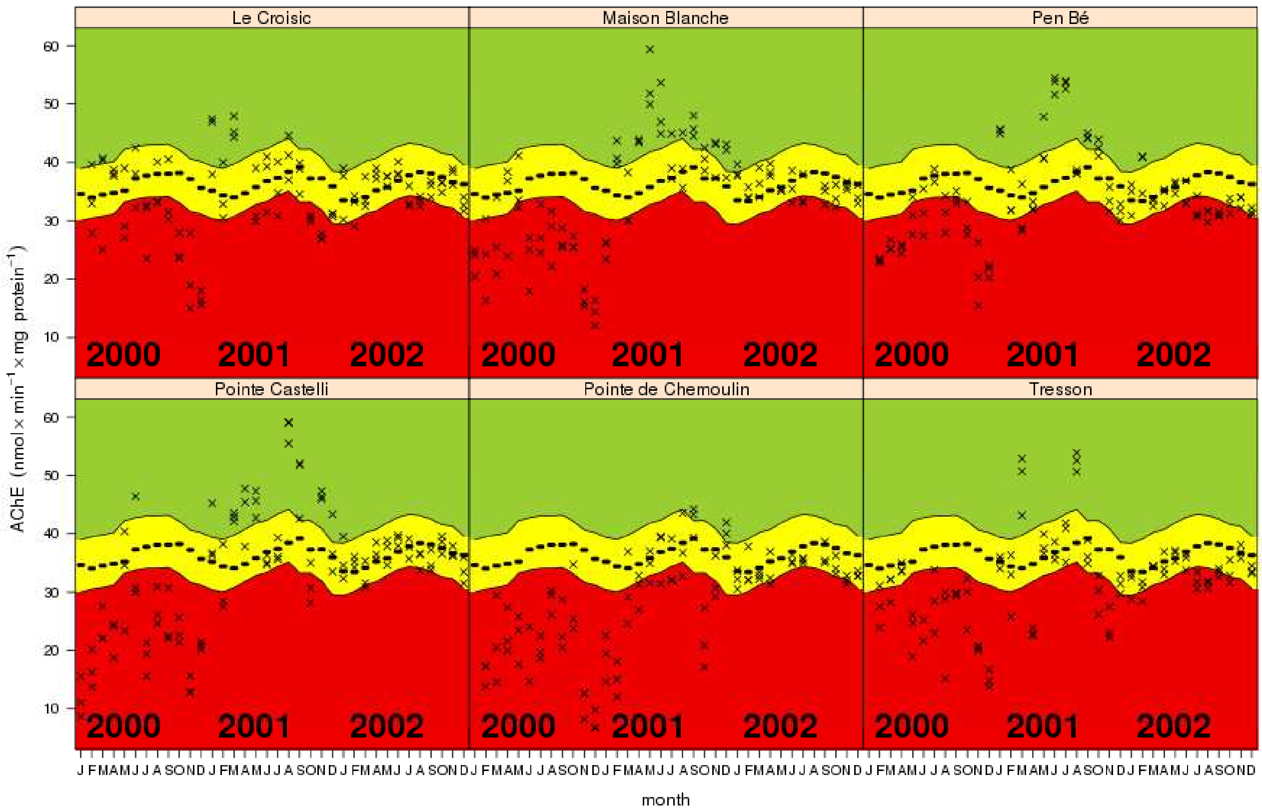

Figure 4: Classification of the AChE activities following the three color classes of neurotoxic effects and comparison of the six stations during the acute (2000) and the period of the restoration (2001-2002).

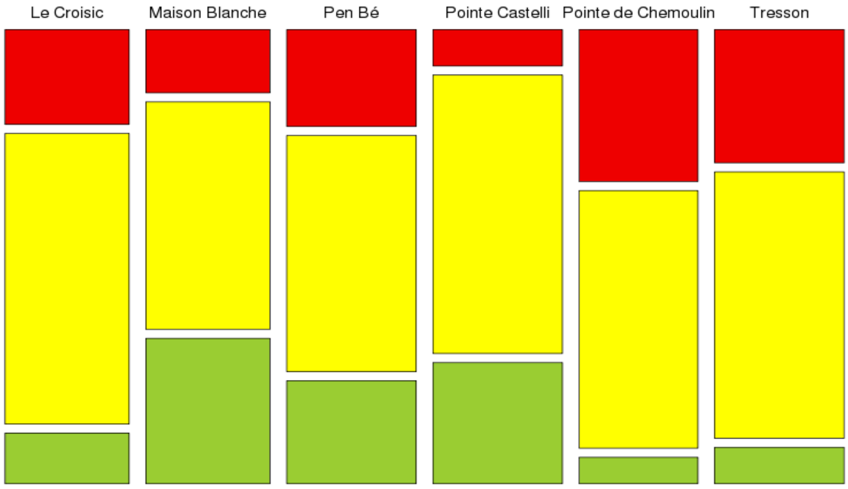

Figure 5: Graphic representation of the color code following the model of three classes of effects (red : bad ; orange : moderate and green: good) between six stations and during the period of the restoration (2001-2002).

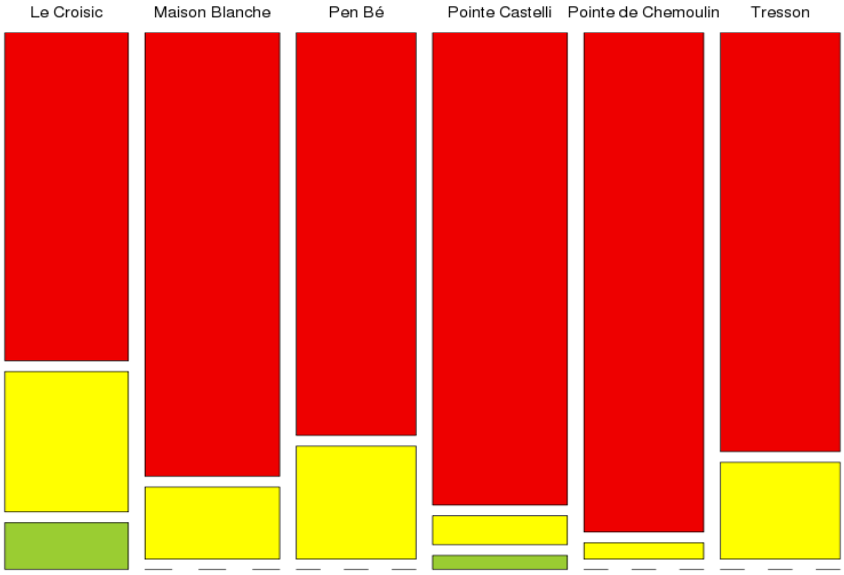

Fig 6 : Graphic representation of the color codes following three classes of effects (Red : bad ; yellow : moderate and green: good) between six stations and during the acute period in 2000.

Tables and legends :

2001	Temp °C	Mean	SD	2002	Temp.°C	Moyenne	SD
January	9.7	34.4	10.41	January	7.65	33.42	3.48
February	9	34.78	9.06	February	9.13	34.13	3.43
March	10.5	34.76	8.53	March	11.5	35.25	2.07
April	12.67	35.81	8.59	April	12.6	35.77	2.51
May	14.9	36.86	7.86	May	15	36.91	1.27
June	15.97	37.37	7.73	June	16.95	37.83	2.31
July	18.18	38.42	7.19	July	18.06	38.36	2.52
August	19.75	39.16	8.5	August	17.6	38.14	2.12
September	15.75	37.26	5.11	September	16.34	37.55	2.16
October	15.8	37.29	7.66	October	14.37	36.61	2.32
November	12.95	35.94	8.23	November	13.7	36.29	2.3
December	7.9	33.54	4.88	December	10.15	34.61	1.42

Table 1: Estimated values of AChE for each monthly temperatures used for the background response level calculation during the period of restoration after the wreck of the Erika (AChE = 29.79630 + (0.47423 x Temperature). Temperatures supplied by Ifremer/quadrige/Rephy

Classes	Pen Bé	Pointe Castelli	Le Croisic	Pointe de Chemoulin	Maison Blanche	Tresson
	16 (23.19%)	6 (8.33 %)	15 (21.74%)	23 (33.33%)	10 (13.89%)	22 (30.56%)
	39 (56.52%)	46 (69.70%)	46 (66.67%)	39 (56.52%)	36 (54.55%)	44 (66.67%)
	17 (23.61%)	20 (27.78%)	8 (11.11%)	4 (5.56%)	23 (31.94%)	6 (8.33%)

Table 2 : Frequencies (Number of AChE values) and percentages (%) determined for each class into the six stations in 2001 and 2002.

Classes	Pen Bé	Pointe Castelli	Le Croisic	Pointe de Chemoulin	Maison Blanche	Tresson
	25 (0.76%)	33 (0.92 %)	21 (0.64%)	31 (0.94%)	31 (0.86%)	26 (0.72%)
	7 (0.19%)	2 (0.06%)	9 (0.25%)	1 (0.03%)	5 (0.16%)	6 (0.19%)
	0 (0.00%)	1 (0.03%)	3 (0.09%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

Table 3 : Frequencies (Number of AChE values) and percentages (%)determined for each class into the six stations in 2000.