Biological effects of contaminants:
Cholinesterase inhibition by organophosphate and carbamate compounds

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td>1 ORGANOPHOSPHATES AND CARBAMATES</td>
<td>1</td>
</tr>
<tr>
<td>2 ACETYLCHOLINESTERASE</td>
<td>1</td>
</tr>
<tr>
<td>3 ACETYLCHOLINESTERASE INHIBITION MEASUREMENTS</td>
<td>2</td>
</tr>
<tr>
<td>3.1 Extraction</td>
<td>2</td>
</tr>
<tr>
<td>3.2 Protein Determination</td>
<td>2</td>
</tr>
<tr>
<td>3.3 AChE Activity</td>
<td>2</td>
</tr>
<tr>
<td>3.3.1 Determination of AChE activity</td>
<td>2</td>
</tr>
<tr>
<td>3.3.2 Calculation of AChE activity</td>
<td>3</td>
</tr>
<tr>
<td>3.4 Species Selection and Sampling Conditions</td>
<td>4</td>
</tr>
<tr>
<td>3.5 Example of Field Data</td>
<td>4</td>
</tr>
<tr>
<td>4 INTERPRETATION OF RESULTS</td>
<td>5</td>
</tr>
<tr>
<td>5 REFERENCES</td>
<td>10</td>
</tr>
</tbody>
</table>
Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds

Gilles Bocquene and François Galgani


Abstract

The recent development of biomarkers based on the study of biological responses of organisms exposed to chemical contaminants has provided the biochemical tools essential to the implementation of programmes for monitoring the biological effects of contaminants. At the initiative of ICES and UNESCO-IOC study groups, several international workshops have advocated the development of biochemical methods for contaminant monitoring programmes in the marine environment.

Among the major contaminants in the marine environment, insecticides have the potential to cause ecotoxicological effects because of their strong persistence (e.g., organochlorines) or high toxicity (e.g., organophosphates and carbamates). Although the transfer of these contaminants to the marine environment is generally diffuse and chronic, there are occasions when accidental discharges can cause ecological and economic impacts. An example of such an event was the loss of several tonnes of carbamates (furathiocarb) from a ship at sea and their subsequent impact on the French coast in December 1993 and January 1994. A biomarker to monitor specifically the effects of organophosphates and carbamates is needed to facilitate the assessment of the discharge of these substances into the marine environment.

Acetylcholinesterase (AChE/EC 3.1.1.7.) inhibition has been used as a biomarker of the effects of organophosphate and carbamate compounds (Coppage and Braidech, 1976; Nemcsok et al., 1985; Zinkl et al., 1987; Day and Scott, 1990). The existence of extremely low thresholds for induction of inhibitory effects on AChE suggests that detection is possible after exposure to insecticide concentrations of around 0.1 to 1 μg l⁻¹ (Klaverkamp and Hobden, 1980; Habig et al., 1986). Data concerning the levels of these contaminants in the different marine compartments are scarce, but studies on sediment and living matter have revealed concentrations above the induction threshold (Barcelo et al., 1991).

A few studies on the characterization of AChE from different marine species have been published (Coppage and Braidech, 1976; Bocquene et al., 1990). Other work concerning the preparation, storage, and handling of samples is also available (Finlayson and Rudnicki, 1985). This paper describes a method for the determination of acetylcholinesterase in invertebrate and vertebrate tissues and the use of this technique in monitoring.

Key words: Acetylcholinesterase, organophosphates, carbamates
ORGANOPHOSPHATES AND CARbamATES

Organophosphates and carbamates are mainly used in agriculture as insecticides; in addition, they are used as biocides in household products. Both organophosphates and carbamates are widely used and are characterized as being acutely toxic, but relatively non-persistent, compounds. More than 100 different organophosphate molecules and fifty carbamate molecules are known. Organophosphates such as parathion, malathion, fenitrothion, phosalone, dichlorvos (vapona), and chlorpyrifos and carbamates such as carbaryl, carbofuran, and propoxur are some of the most widely used insecticides. The annual consumption of organophosphates and carbamates reached 3,000 tonnes in France in 1989. Organophosphates and carbamates represent 60 % to 70 % of the total insecticides in use.

Organophosphates and carbamates are toxic to most organisms owing to their ability to inactivate the enzymes known as cholinesterases (ChEs), and especially acetylcholinesterase (AChE), by taking the place of the natural substrate of the enzyme. Depending upon chemical structure and formulation, the toxicity of carbamate and organophosphate insecticides can vary from only slightly to extremely toxic.

ACETYLCHOLINESTERASE

AChE is present in most animals and is responsible for the rapid hydrolytic degradation of the neurotransmitter acetylcholine (ACh) into the inactive products choline and acetic acid. The role of AChE in cholinergic transmission is to regulate the nervous transmission by reducing the concentration of ACh in the junction through AChE-catalysed hydrolysis of ACh. When AChE is inactivated by an organophosphorus or carbamate ester, the enzyme is no longer able to hydrolyse ACh and the concentration of ACh in the junction remains high. Continuous stimulation of the muscle or nerve fiber then occurs, resulting in tetany and eventually paralysis and death. It should be pointed out that there is another type of cholinesterase family in addition to AChE, namely, butyrylcholinesterases.

AChE has the highest specificity for ACh of any other choline ester and butyrylcholinesterase has the highest specificity for butyrylcholine. AChE is commonly found in nervous tissues, brain, red blood cells, and muscle tissues, while butyrylcholinesterase is found in plasma, liver, and pancreas, where its physiological role is not well defined. It must be noted that the two enzymes differ by just one amino acid in the esterasic site and both are inhibited by organophosphates and carbamates.

The presence of AChE has been demonstrated in a variety of tissues of marine organisms including fish muscle and brain, adductor muscle and gills of shellfish and abdominal muscle of crustaceans, the highest activities being found in the brain and muscle of fish and in the muscle of prawn. Molluscs show low activity.

Carbamate insecticides and some organophosphates are direct inhibitors of ChEs. Many organophosphorus compounds contain the thion moiety (=S) rather than the oxon moiety (=O). The thiono-type organophosphates are not potent inhibitors of ChEs, but require metabolic activation by monoxygenase enzymes to form the active oxon analogues. The liver, the brain, and the lungs contain the greatest capacity for bioactivation, and this may be of significance since such tissues are target organs.
Organophosphates are generally considered irreversible inhibitors of ChE activity, whereas carbamates are slowly reversible inhibitors. Aging produces a time-dependent change in certain organophosphate insecticides that results in extremely tight binding to ChE such that it is essentially irreversible.

3 ACETYLCHOLINESTERASE INHIBITION MEASUREMENTS

3.1 Extraction

Extraction is performed on fresh or frozen tissue (0.1 to 1 g) using 0.02 M phosphate buffer (pH 7.0) containing 0.1 % Triton X 100 (Ellman et al., 1961; Lundin, 1967; Wang and Murphy, 1982). The tissue is homogenized 1/1 to 1/4 w/v depending on the species or organ (for example, fish brain tissue has a higher specific AChE activity than muscle) for one minute using an Ultra Turrax.

Extracts are then centrifuged at 10,000 × g for 20 minutes and an aliquot of the supernatant (the 'S9 fraction') is used in the assay. These S9 supernatants can be stored at −20 °C or below without significant loss of activity.

3.2 Protein Determination

The method described by Bradford (1976) can be used for quantitative determination using bovine serum albumin (BSA) as the protein standard. This method is adapted to be used with a microplate reader. For each microplate well, 280 μl of Bradford’s reagent (phosphoric acid, methanol, and Coomassie brilliant blue) is added to 100 μl of an appropriate dilution of the sample. A protein standard curve (1 to 10 μg ml⁻¹) is prepared each time the assay is performed by diluting lyophilized bovine serum albumin with distilled water. Rehydrated protein can be stored up to 60 days at 4 °C. Dilutions of the concentrated Bradford’s reagent (commercially available) with distilled water must be utilized within two weeks. The colour development is stable for one hour. Absorbance is read at 595 nm and the sample concentration is calculated from the standard curve. Results are given as mg ml⁻¹ or μg ml⁻¹ of crude extract.

3.3 AChE Activity

3.3.1 Determination of AChE activity

The method used to measure acetylcholinesterase activity has been described by Ellman et al. (1961); it uses acetylthiocholine iodide (ACTC) as a specific substrate. Ellman’s method is based on the increase of yellow colour produced from thiocholine when it reacts with dithiothreitol (DTNB) ion (equation 1, below) and can be adapted for use with a microplate reader.

\[
\text{acetylthiocholine} \xrightarrow{\text{acetylcholinesterase}} \text{thiocholine} + \text{acetic acid}
\]

\[
\text{thiocholine} + DTNB \rightarrow \text{formation of TNB} \text{ (yellow colour (412 nm))}
\]  

(1)
To each well of the microplate, 340 µl of 0.02 M phosphate buffer (pH 7), 20 µl of 0.01 M DTNB (0.5 mM final concentration) and 10 µl of S9 supernatant are added in succession. After five minutes' incubation to allow the DTNB to react with the sulfhydryl groups of the amino acids in the sample, 10 µl of 0.1 M ACTC (2.6 mM final concentration) is added to begin the enzymatic reaction. (Note: AChE is subject to substrate inhibition at 10 mM final concentration of ACTC, so care should be taken to ensure that the final concentration of 2.6 mM is maintained accurately.) All reagents must be brought to room or controlled temperature prior to use. The enzyme kinetics are monitored on a microplate reader at 412 nm (from 405–420 nm). Wells without enzymatic extract (buffer + DTNB + ACTC) are used to estimate the spontaneous hydrolysis of ACTC. The assay can be run from one to several minutes depending on the reaction rate of the enzyme being measured. This will depend on the tissue or organism under study and the degree of inhibition observed.

Using a microplate, all assays can be easily duplicated. When assays are performed in quadruplicate, a coefficient of variation of 6% is usually obtained, depending on the expertise of the operator.

With regard to storage conditions for the reagents, desiccated ACTC must be stored at temperatures lower than 0 °C and the 0.1 M ACTC stock solution must be prepared in distilled water immediately prior to use.

0.01 M DTNB is prepared in 0.1 M TRIS, pH 8 buffer and can be used for several days when stored at 4 °C.

### 3.3.2 Calculation of AChE activity

The change in absorbance (OD) per minute is determined by subtracting the absorbance due to non-enzymatic hydrolysis of substrate from the absorbance increase per minute measured for the sample. Enzyme activity is then expressed in Units of activity, where one Unit is defined as the amount of enzyme which catalyses the hydrolysis of 1 µmole of acetylthiocholine per minute per mg protein, and is calculated using the following formula (equation 2):

$$AChE\ activity\ (\mu mol\ ACTC\ min^{-1}\ mg\ protein^{-1}) = \frac{\Delta A_{412} \times Vol_T \times 1000}{1.36 \times 10^4 \times lightpath \times Vol_s \times [protein]}$$

where:

- $\Delta A_{412}$ = change in absorbance (OD) per min, corrected for spontaneous hydrolysis
- $Vol_T$ = total assay volume (0.380 ml)
- $1.36 \times 10^4$ = extinction coefficient of TNB (M⁻¹ cm⁻¹)
- $lightpath$ = microplate well depth (1 cm)
- $Vol_s$ = sample volume (in ml)
- $[protein]$ = concentration of protein in the enzymatic extract (mg ml⁻¹).
3.4 Species Selection and Sampling Conditions

The choice of species for use in monitoring is difficult because it depends very much on the problem and the area being monitored. However, the species must be widely distributed, its biology should be well known, preferably it should be sedentary or have a restricted migratory range, and it must show a detectable AChE activity. Furthermore, it is important to know the natural limits of variability in AChE activity in the species of interest, in order that the significance of observed depression in activity can be assessed. Although few factors are known to affect the level of AChE activity, knowledge of possible variations related to sex, size, state of gonadal maturation and the influence of seawater temperature should be systematically determined. Most benthic finfish (muscle and brain), crustaceans (abdominal muscle), and bivalve molluscs (adductor muscle and gills) offer the potential for monitoring AChE activity. Assays must be performed on individuals of known age (size) and sex, and all the samples from one site should be analysed under the same conditions at the same time. The recommended number of samples required is a minimum of six to ten animals per site.

3.5 Example of Field Data

The ICES/IOC International Workshop on Biological Effects of Contaminants, that took place in Bremerhaven, Germany during March 1990, provided an opportunity to test AChE inhibition as an index of marine contamination.

One of the two locations sampled was the German Bight transect that consists of seven stations running northwest from a point off the mouths of the Weser and Elbe rivers (Stations 1 and 3) to the most offshore station located over the Dogger Bank (Station 9), traversing a known contaminant gradient (Stebbing and Dethlefsen, 1992).

The specific activity of AChE was determined for the muscle of ten dabs (*Limanda limanda*) from each station (Galgani *et al.*, 1992). The AChE activity varied according to the expected contamination gradient (Figure 1). The most marked inhibitory effects were noted at Station 3, while the activity increased from Station 3 to Station 8, where the highest activity was found. AChE activity decreased at Station 9 when compared with Station 8, but it is not known whether this decrease is within the natural limits of variability of dab. The effects observed may indicate the presence of neurotoxic molecules known to be the strongest cholinesterase inhibitors. Some very limited data exist on the presence of these compounds in the North Sea. Up to 0.5 µg l\(^{-1}\) of methyl parathion has been measured in the mouth of the Elbe River (Figure 2), but it is not known whether the much lower levels observed offshore could account for the effects observed on the Bremerhaven transect.

Acetylcholinesterase inhibition has mainly been used to monitor the effects of insecticides in terrestrial environments, forests, and fresh water (Williams and Sova, 1966; Gelman and Herzberg, 1979; Day and Scott, 1990). For example, Payne *et al.* (1994) have observed a 50 % inhibition of brown trout AChE captured in a polluted urban river. In the marine environment, the measurement of AChE inhibition in problem-oriented monitoring is now used to control the impact of dichlorvos used locally by salmon farmers as a parasitic control agent (McHenery *et al.*, 1991, 1996).
INTERPRETATION OF RESULTS

In many cases, the natural variability of AChE is not directly related to age, sex, or reproductive period in mature fish, although certain early stages are known to show high cholinesterase activities. Two of the most important factors affecting AChE activity are the temperature of the environment from which the organism was sampled and the temperature of the assay (Hogan, 1970). Careful and consistent attention should be given to the control of these two variables. In the field, samples should be obtained synchronously within a time scale of several days and in comparable climatic conditions, especially with regard to the temperature of the sea water. Only stations in the same geographical area should be compared and the temperature of the water at the time of sampling should be reported.

Most organophosphate and carbamate compounds show very high affinity for AChE. Recent studies (see Table 1) indicate high inhibitory activities of several organophosphates and carbamates on AChE extracts of marine organisms (Bocquene et al., 1995). Thus, the IC₅₀ (concentration inducing 50% inhibition) of carbofuran on the AChE of common prawn (Palaemon serratus) was 1.1 x 10⁻⁸ M (2.4 µg l⁻¹) and 2.8 x 10⁻⁸ M (6.2 µg l⁻¹) for the AChE of dragonet (Callionymus lyra). Carbaryl (a carbamate) and phosalone (an organophosphate) induced a significant inhibitory effect on the AChE of common prawn at a concentration of 0.1 µg l⁻¹ after 29 days of contact.

Table 1. Concentration values in M (mean ± s.d.) of several organophosphate and carbamate insecticides causing 50% AChE inhibition (IC₅₀) after a one-hour incubation. Numbers in parentheses indicate the number of replicates.

<table>
<thead>
<tr>
<th></th>
<th>Dragonet</th>
<th>Sole</th>
<th>Prawn</th>
<th>Oyster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbaryl</td>
<td>3.0 x 10⁻⁷ ± 0.7 x 10⁻⁷ (6)</td>
<td>7.8 x 10⁻⁶ ± 0.3 x 10⁻⁶ (3)</td>
<td>1.7 x 10⁻⁷ ± 0.6 x 10⁻⁷ (4)</td>
<td>1.5 x 10⁻⁷ ± 0.5 x 10⁻⁷ (5)</td>
</tr>
<tr>
<td>Carbofuran</td>
<td>2.8 x 10⁻⁸ ± 0.7 x 10⁻⁸ (7)</td>
<td>8.0 x 10⁻⁷ ± 0.5 x 10⁻⁷ (3)</td>
<td>1.1 x 10⁻⁸ ± 0.3 x 10⁻⁸ (5)</td>
<td>1.5 x 10⁻⁸ ± 0.5 x 10⁻⁸ (5)</td>
</tr>
<tr>
<td>Dichlorvos</td>
<td>2.3 x 10⁻⁸ ± 0.4 x 10⁻⁸ (5)</td>
<td>3.1 x 10⁻⁷ ± 0.8 x 10⁻⁷ (6)</td>
<td>1.1 x 10⁻⁶ ± 0.2 x 10⁻⁶ (5)</td>
<td>7.3 x 10⁻⁸ ± 1.0 x 10⁻⁸ (6)</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>2.6 x 10⁻⁵ ± 0.5 x 10⁻⁵ (4)</td>
<td>1.2 x 10⁻⁴ ± 0.3 x 10⁻⁴ (5)</td>
<td>&gt; 10⁻⁴</td>
<td>1.4 x 10⁻⁴ ± 0.2 x 10⁻⁴ (5)</td>
</tr>
<tr>
<td>Phosalone</td>
<td>6.2 x 10⁻⁷ ± 1.5 x 10⁻⁷ (5)</td>
<td>2.5 x 10⁻⁶ ± 0.7 x 10⁻⁶ (4)</td>
<td>&gt; 10⁻⁴</td>
<td>&gt; 5 x 10⁻⁴</td>
</tr>
</tbody>
</table>
Figure 1(a). Position of sampling sites and currents along the German Bight.
Figure 1(b). Specific activity of acetylcholinesterase in muscle of dab (*Limanda limanda*) caught along the German Bight transect (stations shown in Figure 1(a)). Results (mean ± s.d.) are expressed in absorbance units per mg of protein. Ten fish were sampled per station and four measurements were made per sample.

Although numerous insecticide formulations contain several different molecules, there are few data on the potential additivity or synergism of their effects on AChE. A recent study (Bocquene *et al.*, 1995) showed that the effects of the combinations, by pair, of five organophosphates and carbamates were synergistic, i.e., the total inhibitory effect was greater than the addition of the respective effects of each compound (see Table 2). The strongest synergy was obtained with organophosphate/carbamate associations rather than organophosphate/organophosphate or carbamate/carbamate associations. Moreover, synergistic effects were closely related to the length of time over which the enzymatic extract was incubated with the inhibitory mixtures; they tended to be stronger as the incubation time increased. For many combinations, for example, carbaryl/phosalone, dichlorvos/carbofuran, or phosalone/carbofuran, inhibitory effects were enhanced tenfold.
Figure 2. Methyl parathion (ng l⁻¹) in the mouth of the Elbe River and in water from the German Bight in January 1987. [Source: Deutsches Hydrographisches Institut, 1987 (Jahresbericht).]
Table 2. Joint toxicity of organophosphorus and carbamate mixtures on AChE from different marine organisms. Results are given in Toxic Units ± s.d. of four determinations, nd = values not determinable.

<table>
<thead>
<tr>
<th></th>
<th>Carbofuran</th>
<th>Dichlorvos</th>
<th>Fenitrothion</th>
<th>Phosalone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carbaryl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dragonet</td>
<td>0.72 ± 0.06</td>
<td>0.50 ± 0.04</td>
<td>0.58 ± 0.05</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>Sole</td>
<td>0.74 ± 0.06</td>
<td>0.38 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>Prawn</td>
<td>0.76 ± 0.07</td>
<td>0.70 ± 0.05</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Oyster</td>
<td>0.72 ± 0.05</td>
<td>0.72 ± 0.05</td>
<td>0.56 ± 0.04</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Dichlorvos</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dragonet</td>
<td>0.42 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sole</td>
<td>0.24 ± 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prawn</td>
<td>0.34 ± 0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oyster</td>
<td>0.76 ± 0.08</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fenitrothion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dragonet</td>
<td>0.28 ± 0.02</td>
<td>0.66 ± 0.06</td>
<td></td>
<td></td>
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<tr>
<td>Sole</td>
<td>0.45 ± 0.03</td>
<td>0.54 ± 0.03</td>
<td></td>
<td></td>
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<tr>
<td>Prawn</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oyster</td>
<td>0.45 ± 0.04</td>
<td>0.68 ± 0.06</td>
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<td></td>
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<tr>
<td><strong>Phosalone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dragonet</td>
<td>0.30 ± 0.02</td>
<td>0.20 ± 0.01</td>
<td>1.10 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Sole</td>
<td>0.22 ± 0.01</td>
<td>0.58 ± 0.03</td>
<td>0.25 ± 0.02</td>
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</tr>
<tr>
<td>Prawn</td>
<td>nd</td>
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<td>nd</td>
<td></td>
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<tr>
<td>Oyster</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

The type of joint action was determined according to the following formula:

\[ xTU_A + yTU_B = 1 \ TU_{(A+B)} \]

where TU is the toxic unit, one unit of toxicant represents the IC_{50} of a cholinesterase inhibitor (organophosphate or carbamate), \( A \) and \( B \) represent the AChE inhibitors (organophosphate and carbamate), and \( x \) and \( y \) are the proportional toxic units of \( A \) and \( B \) present in the mixture (e.g., 0.5A + 0.5B, in this case \( x = 0.5 \) and \( y = 0.5 \)).

If the addition of half the concentration of toxicant \( A \) necessary to produce IC_{50} and half the concentration of toxicant \( B \) necessary for the same response just causes the IC_{50}, \( A \) and \( B \) are exactly additive; if it causes more than the expected response, i.e., more than 50% inhibition, the joint action is synergistic. (The lower the TU, the higher the synergistic action of the two inhibitors.)

Thus:

if \( x + y = 1 \), the joint action is additive;
if \( x + y < 1 \), the joint action is synergistic (the lower the total \( x + y \) is, the higher the synergistic action);
if \( x \) or \( y > 1 \), the action is antagonistic.

ICES Techniques in Marine Environmental Sciences, No. 22
Organophosphate and carbamate insecticides are strong inhibitors of acetylcholinesterase, but other chemicals can result in \textit{in vitro} inhibition. Among inorganic chemicals, arsenate and arsenite ions (4–11 mg l\(^{-1}\)), mercuric chloride (6 mg l\(^{-1}\)), and copper chloride show an inhibitory effect on the AChE activity of common prawn, sole, and dragonet, but no inhibitory effect was observed with zinc, cadmium, and tributyltin chlorides, even at 10 mg l\(^{-1}\). In the same way, cholinergic-acting neurochemical agents (such as atropine and nicotine) have a moderate inhibitory effect (Olson and Christensen, 1980). But, if some of these compounds are not important marine contaminants (neurochemical agents), the concentrations required to cause inhibition of AChE from those that are known to be toxic (arsenic ions) are so large that concentrations this high are not likely to be found in the environment, even in sediments. Indeed, concentrations of organophosphates and carbamates required to cause 50 % reduction of enzyme activity (IC\(_{50}\)) are in the range of 10\(^{-5}\) to 10\(^{-9}\) M. No inhibitory effect was observed for other pesticides such as organochlorines (DDT, lindane) or herbicides (atrazine, isoproturon).

Some algal toxins have been reported to be strong AChE inhibitors. These anatoxins are mainly produced by algal blooms of cyanobacteria: \textit{Microcystis aeruginosa}, \textit{Anabaena flos-aquae}, and \textit{Aphanizomenon flos-aquae}. These three species are freshwater organisms and, although they can be found in brackish waters (up to a salinity of 8), they have never been found in marine waters. Anatoxin-a(s) is a potent anticholinesterase agent but it is only released by the cyanobacteria \textit{Anabaena flos-aquae} and not by any other known species (Carmichael, 1994). Nevertheless, to avoid confounding factors it is recommended that the presence of any algal blooms and their identity should be noted when the samples are collected.

Unfortunately, few data on contamination levels of organophosphates and carbamates in the marine environment are available. Such information appears to be essential to link the variation in cholinesterase activity with the presence of organophosphates and carbamates.

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No. 8  Soft bottom macrofauna: Collection and treatment of samples
No. 9  Sediments and suspended particulate matter: Total and partial methods of digestion (videotape available)
No. 10 Organic halogens: Determination in marine media of adsorbable, volatile, or extractable compound totals
No. 11 Biological effects of contaminants: Oyster (Crassostrea gigas) embryo bioassay
No. 12 Hydrocarbons: Review of methods for analysis in seawater, biota, and sediments
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