Acetylcholinesterase activity in seabirds affected by the Prestige oil spill on the Galician coast (NW Spain)

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Abstract

In November 2002, the tanker Prestige broke in two and sank at the bottom of the ocean spilling about 70,000 t of fuel oil, which reached the coast of Galicia. It was considered the largest spill in maritime history, greatly affecting marine and related avian species. The spilled fuel oil contained high concentrations of polycyclic aromatic hydrocarbons (PAHs). Many species were affected and were found dead, although ongoing research is still being carried out on the sublethal effects. In this sense, little is known about the action of PAHs on Cholinesterase activity in seabirds. Consequently, the purpose of this study was to provide more information on the neurotoxicity of fuel oil on the seabirds most affected by the Prestige accident: common guillemot, Atlantic puffin and razorbill. On the other hand, data on normal values of acetylcholinesterase (AChE) activity were obtained to supply non-exposed values in seabirds. The oil spill produced a clear inhibitory effect on brain AChE activity in common guillemot (16%, \(p \leq 0.01\)) and razorbill (22%, \(p \leq 0.01\)), but not in Atlantic puffin (4%). Physiological levels of brain AChE, expressed in nmol acetylcholine hydrolysed min⁻¹ mg⁻¹ protein were similar in non-exposed common guillemot (388.6±95.0) and Atlantic puffin (474.0±60.7), however, razorbill values were higher (644.6±66.9).

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1. Introduction

Most accidental oil spills occur when oil is being transported by tankers. Unfortunately, the Galician coast (Northwest of Spain) has suffered several oil tanker accidents due to the roughness of its sea. The danger of this coast makes Galicia the European region with the greatest number of this kind of disaster. Some examples of tankers that have sunk in this coast are Polymander (1970), Erkowit (1973), Urquiola (1976), Andros Patria (1979), Aegean Sea (1992), and more recently the Prestige (2002). This latter accident represents one of the largest environmental catastrophes in European navigation, as the tanker discharged about 70,000 t of fuel oil, about twice the size of the Exxon Valdez spill in Alaska (1989). Thus, contamination produced by the Prestige spill was extensive. According

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to the report presented by the Spanish Government to the International Oil Pollution Compensation Funds, 70% of Galician beaches were affected by the oil (IOPC Funds, 2003); the oil formed a large slick on the surface of the water that washed onto beaches and rocks along the coast, affecting the sea bed, this process being accelerated by tide movements and ocean currents (Pérez-Cadahía et al., 2004).

Marine birds, which are found to be among the most conspicuous victims of oil spills at sea, experienced substantial mortality as a result of the Prestige accident: 9757 carcasses were collected throughout the spill-affected areas of Galician coasts from November 2002 to August 2003 (SEO/Birdlife, 2003). Apart from mortality, oil has been shown to have a great variety of deleterious effects on birds, including physical and physiological effects together with those on survival. Thus, external physical applications caused loss of buoyancy, inability to fly, inflammation, and increased basal metabolic rate (Hartung and Hunt, 1966; Lee et al., 1982). Short-term effects of oil ingestion also included reduced rate of growth (Peakall et al., 1982). In addition, several authors have observed long-term effects on reproductive success through lowered hatchability and altered yolk structure of eggs (Grau et al., 1977), and on the development and survival of chicks that were slow and reduced, respectively (Trivelpiece et al., 1984). Likewise, long-term effects included extensive damage to the liver, kidneys, and intestine (Khan and Ryan, 1991; Patton and Dieter, 1979; Fry and Lowenstein, 1985).

Fuel analyzed by Spanish authorities had a low solubility, high adherence, viscosity and density, low dispersion capacity and slow degradation. It contained high levels of several polycyclic aromatic hydrocarbons (PAHs) and heavy metals. Regarding hydrocarbons, its main composition consisted of 50% aromatic hydrocarbons, 20% saturated hydrocarbons and 35% resins and asphalthene, according to the analysis made by the Superior Centre of Scientific Research of Spain (CSIC, 2003). Thereby, it was a relatively low volatile oil (5–10%), due to its greater content of high molecular weight hydrocarbons.

PAHs released into the marine environment with the Prestige oil spill have contributed significantly to the environmental toxicity of the fuel oil. Apart from the large number of deaths that occurred, aquatic organisms exposed to lower concentrations may exhibit biochemical, physiological, and/or behavioural responses. It is known that some of the possible biomarkers of oil exposure in free-ranging species are molecules that are induced in response to a contaminant and presumably can be related to the health or fitness of the wildlife (Hugget et al., 1992; Peakall and Shugart, 1993). Thus, several biomarkers, such as cytochrome P450 induction, peroxisome proliferation or lysosomal alterations have been applied in the assessment of the impact of oil spillages on several organisms (Porte et al., 2000; Cajaraville et al., 1997, 2000). In addition, physiological responses, such as feeding impairment and reduction of the scope for growth, have also been successfully applied as ecological relevant sublethal responses to PAHs exposure, providing important insights on growth and reproductive disruption at population level (Maltby et al., 2001).

Acetylcholinesterase (EC 3.1.1.7., AChE), in nervous tissue, is responsible for acetylcholine degradation, one of the most important neurotransmitters in either the central or peripheral nervous system. AChE activity has been used as an enzymatic biomarker of neurotoxicity caused by pesticides; AChE activity is specifically inhibited by the presence of organophosphate and carbamate, but recently it has been discovered that other chemicals have similar actions on AChE response. Within environmental chemicals, some heavy metals and petroleum-derived products have been reported as AChE inhibitors in marine organisms (Chamber et al., 1978; Sheehan et al., 1991; Bocquené et al., 1995; Martínez-Tabche et al., 1997; Mora et al., 1999; Akcha et al., 2000; Moreira et al., 2004, 2005; Cunha et al., 2005), and in “in vitro” studies (Kang and Fang, 1997; Jett et al., 1999).

Nevertheless, little is known about the effects of PAHs on the physiology of the nervous system in seabirds and/or on the AChE activity response to incidental exposures. Kang and Fang (1997) have been able to show that several PAHs, commonly found in the environment, such as pyrene (PY), benzo(a)pyrene (BP), fluoranthene (FL) and anthracene (AN) are weaker inhibitors of AChE activity “in vitro”. These authors have established the hypothesis that those PAHs with more aromatic rings are more potent inhibitors of AChE activity. Therefore, “in vivo” research is necessary to determine the toxicological relevance of these “in vitro” findings. AChE activity determination in seabirds following PAHs exposure has not yet been assessed. Consequently and in order to evaluate the biological significance of the exposure and/or contamination of marine ecosystem following the Prestige oil spill, measurement of AChE activity was performed on the three species of seabirds more severely affected by the oil spill (common guillemot—Uria aalge, Atlantic puffin—Fratercula arctica and razorbill—Alca torda), according to SEO/Birdlife (2003).
The objectives of this study were (1) to provide information on neurotoxicity of fuel oil in seabirds under natural conditions, by assessing AChE activity in seabirds affected by the Prestige oil spill on the Galician coast (NW Spain), (2) to study the response of this enzymatic activity as an indicator of exposure to oil and (3) to contribute to physiological levels of AChE activity in the avian species studied.

2. Materials and methods

Ecologically relevant seabirds, such as common guillemot (U. aalge), Atlantic puffin (F. arctica) and razorbill (A. torda), which fed, rested and bred on waters and shores of Galicia, constituted the main group of birds affected by the spill, since this episode of contamination occurred during one of the main migratory periods of these birds, which came from the British Isles, resulting in a large number of birds being found in the area at that precise moment. As it is known, these gregarious marine birds spend most of their time floating on the water surface where they are in contact with oil, and according to Rocke (1999) and Murphy et al. (1997), seabirds such as common guillemots and puffins are particularly susceptible to contamination from oil spills.

Nevertheless, sampling of oceanic wild seabirds can be extremely complicated. It also poses serious ethical problems, especially with rare or threatened species. This leads to the fact that most studies are conducted with seabirds that have died rather than with live specimens (Spahn and Sherry, 1999; Pérez-López et al., 2006). Thus, seabirds, such as common guillemot (n=24), Atlantic puffin (n=22) and razorbill (n=30) were collected dead or moribund on the beaches. They were referred to the Wildlife Recovery Centres from the end of November 2002 to May 2003, and thereafter were sent to the Toxicology Laboratory of the Veterinary Science Faculty (University of Extremadura, Cáceres, Spain). Non-exposed marine birds (n=20/group) were collected before the contamination episode at the same geographical area. Carcasses from both exposed and non-exposed birds were frozen at −80 °C after collection until AChE activity was determined. In the laboratory, birds were decapitated, the whole brain was immediately removed and homogenized in Tris buffer (0.1 M; 1% Triton X100; pH=8). Homogenates were centrifuged at 10000 ×g for 30 min at 4 °C (Avanti® 30, Beckman Coulter Inc., Fullerton, CA).

AChE activity in the supernatant was determined spectrophotometrically, at 405 nm, 42 °C, according to the Ellman method (Ellman et al., 1961) adapted to microplate, following the general procedure described by Thompson (1999). AChE activity was determined in brain homogenate supernatants and the activity was expressed in Units (U) per mg of protein (1 U is a nmol acetylcholine hydrolysed per min). Supematant protein content was quantified by the bicinchoninic acid method (kit from Sigma; BCA-1 and B 9643) adapted to microplate using bovine serum albumin (BSA) as standard. A FL600 microplate reader was used (Bio-Tek® FL-600, Winooski, VT) to these purposes, since Tor et al. (1994) demonstrated the ability of a 96-well microplate reader for routine determinations of enzyme activity, giving results that are sensitive, accurate, reproducible enough, and widely applicable.

A statistical package (SPSS for Windows, V. 13.0) was used to analyze the results. Data were tested for normal distribution (Kolmogorov–Smirnov normality Test) and verified for homogeneity of variance (Levene Test) (Zar, 1996). According to the results of the previous tests, differences between non-exposed and exposed groups for each species were assayed by using the parametric T Student-Test or the non-parametric U-Mann-Whitney Test for independent samples. Differences in activity between the three non-exposed species of birds were compared by one-way analysis of variance (ANOVA) and statistical different interspecies were identified by Bonferroni test (Zar, 1996). The level of significance selected was p ≤ 0.01.

3. Results and discussion

As can be seen in Table 1, AChE activity was significantly depressed during the contamination episode (t = -2.75, df=42, p ≤ 0.01) in common guillemot with regards to non-exposed values for this bird. Major AChE depression was found in razorbill, in which a statistical difference could also be detected with regards to their non-exposed congeners (t = -6.47, df=48, p ≤ 0.01). Apart from the statistical study of AChE activity, considerations have to be made on the levels of enzyme depression as well. In this sense, AChE was depressed by 16% and 22% in exposed common guillemot and razorbill, respectively. The mean value in Atlantic puffin exposed group was only 4% lower than its control group, and this difference was not statistically significant.

Values of brain AChE activity in non-exposed birds ranged from 228.5 to 533.3 U per mg of protein in common guillemot, with similar values in Atlantic puffin (373.9–577.9), but higher values were found in razorbill (542.6–752.2). The coefficients of variation were 24.4%, 12.8% and 10.4% for common guillemots, puffins and razorbills, respectively. Comparing the brain
AChE activity between these groups of non-exposed birds, the ANOVA test showed significant differences among the three avian species ($F=59.35$, $p<0.001$). When realizing the post hoc Bonferroni test, statistical differences were observed between the three seabird species considered ($p<0.01$), the lower and higher mean values corresponding to common guillemots and razorbill samples respectively (Table 1).

In our survey, razorbill and common guillemot AChE activity was apparently affected by the oil spill, since a statistical significance ($p \leq 0.01$) was attained with regards to non-exposed birds. Ludke et al. (1975) suggested that $\geq 20\%$ inhibition of brain AChE activity is indicative of exposure to a ChE-inhibiting pesticide, and inhibitions greater than 50% are enough to diagnose cause of death. The last JMPR Meeting (1998) supported this criterion for anticholinesterasic compounds and considered that statistically significant inhibition by 20% or more represents a clear toxicological effect.

The level of enzymatic depression for razorbill was approximately 22%, which surpasses the trigger value proposed. Consequently, it can be stated that the oil spill produced an inhibitory effect on AChE activity in this kind of bird. This result is in accordance with those obtained in several field and laboratory studies where evidence that PAHs may inhibit the AChE activity in marine organisms such as oysters, mussels and sea-urchins were shown (Chamber et al., 1978; Sheehan et al., 1991; Bocquené et al., 1995; Martinez-Tabche et al., 1997; Akcha et al., 2000; Moreira et al., 2004, 2005; Cunha et al., 2005), although the mechanisms of this inhibition are not still clear.

A statistically significant depression of AChE activity was also observable for common guillemot, but accounted for less than 20% of inhibition regarding non-exposed birds, therefore a definitive conclusion was not possible due to lack of further data on clinical findings, such as diarrhoea, behaviour or motor activity alterations. In this sense, the JMPR Meeting (1998) considered that statistically significant inhibition of less than 20% or statistically insignificant inhibition above 20% indicates that a more detailed analysis of the data should be undertaken.

Balseiro et al. (2005) reported severe dehydration, diarrhoea and emaciation as main findings in most of the birds affected by Prestige spill. For the authors, diarrhoea was a consequence of mucosal irritation by the presence of a variety of irritants in the ingested oil, including naphthalenes, a type of fuel spilled by the Prestige (CSIC, 2003; IFREMER, 2003). The irritant effect of PAHs could have reduced the absorption of nutrients and contributed to cachexia and diarrhoea, but taking into account our results, it is possible that AChE inhibition contributed, at least in part, to increase the severity of oil spill clinical findings.

Considering the complex composition of fuel oil, it is difficult to attribute the effects to specific substances. Casellas et al. (1995) reported that hydrocarbons were the predominant family of compounds found in crude oil (50%–98%), therefore they are the most important group of environmental contaminants, either for their abundance or for their presence in several environmental compartments. In order to investigate which compound(s) could be responsible for enzyme depression in razorbill and common guillemot, two independent reports on the Prestige oil spill chemical characterization were consulted, one from CSIC (Spain) and another from IFREMER (France). The results are summarized in Table 2.

The main aromatic hydrocarbons present in the fuel—oil were diaromatic hydrocarbons, such as naphthalene and its alkyl derivates. Among the polyaromatic hydrocarbons with 3 rings, the presence of phenanthrene and its alkyl derivates was noticeable. Other polyaromatic hydrocarbons with more than 3 rings, such as fluoranthene and chrysene, together with their alkyl derivates were also main components in the fuel—oil. With similar characteristics to aromatic hydrocarbons, the aromatic

### Table 1

<table>
<thead>
<tr>
<th>Descriptive statistics</th>
<th>Common guillemot</th>
<th>Atlantic puffin</th>
<th>Razorbill</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-exposed ($n=20$)</td>
<td>Exposed ($n=24$)</td>
<td>Non-exposed ($n=20$)</td>
</tr>
<tr>
<td>Mean</td>
<td>388.6*</td>
<td>325.0*</td>
<td>474.0*</td>
</tr>
<tr>
<td>SD</td>
<td>95.0</td>
<td>56.3</td>
<td>60.7</td>
</tr>
<tr>
<td>CV (%)</td>
<td>24.4</td>
<td>17.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Median</td>
<td>397.0</td>
<td>324.1</td>
<td>453.2</td>
</tr>
<tr>
<td>Minimum</td>
<td>228.5</td>
<td>219.6</td>
<td>373.9</td>
</tr>
<tr>
<td>Maximum</td>
<td>533.3</td>
<td>417.6</td>
<td>577.9</td>
</tr>
</tbody>
</table>

*Significant difference between non-exposed and exposed birds within each species ($p \leq 0.01$; Student $t$ test).

**Different characters between non-exposed groups indicate statistically significant interspecies differences ($p<0.01$; post hoc Bonferroni test).
heterocyclic with a sulphur group (dibenzothiophene) was detected.

Taking into account the information supplied by CSIC and IFREMER, none of the main components of spill were apparently identified as clear cholinesterase inhibitor agents, nevertheless an "in vitro" study, carried out by Kang and Fang (1997), indicated that PAHs with 3 or more aromatic rings showed negative effects on AChE activity. In the Prestige oil spill, the majority of the compounds contained 3 or more aromatic rings, excepting naphthalene and derivates, and this fact supports the findings of the present study. Some PAHs are unable to pass through cell membranes in a living organism, due simply to molecule size. In practice, this limit lies around the molecular weight corresponding to 6-ring compounds (E & P Forum, 2002). The findings provided by CSIC and IFREMER on the composition of Prestige fuel oil pointed out that the majority of the components comprised PAHs with less than 6 rings, which can facilitate the transport through biological membranes. In addition, the stability and lipophilicity of PAHs result in their tendency to be concentrated in fatty tissues, like the brain. Thereby, some PAHs may be distributed to the brain and can be related with inhibition of enzymes involved in the metabolism of neurotransmitters, as acetyl cholinesterase, and as a consequence related to impairments in nervous system functions. Tang et al. (2003) also found that although the concentrations of PAHs in brain tissue are much lower than in liver or kidney, their clearance from the brain is apparently much slower than from other tissues. However, a more detailed study would be required to confirm that "in vivo" findings could explain in-vitro results described by Kang and Fang (1997).

The degree of brain AChE inhibition associated with illness or death is influenced by the kind of anti-ChE compound involved and by the species of study. Our survey showed that razorbill was the most affected species, followed by common guillemots, when assessing the AChE depression caused by PAHs. However, Atlantic puffins appeared to be much less sensitive to the inhibitory action of PAHs on AChE activity.

Our study is only a first approach to the problem. Thus, further field studies incorporating a systematic monitoring of wild populations are recommended.

4. Conclusion

The authors suggest that PAHs from oil are unlikely to produce a neurotoxic effect through inhibition of AChE, but in some birds (common guillemots and razorbills) these might have contributed to an enhancement in systemic toxicity. The results obtained in this work showed that the suggested biomarker AChE in the studied seabirds was a non-sensitive indicator of exposure to this kind of environmental pollution.

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