bioassay as a tool to toxic effects of persistent organic pollutants (POPs):

a study with special reference to thiamine deficiency, EROD induction and the bursa of Fabricius

Daphne F. de Roode

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THE CHICKEN EMBRYO BIOASSAY AS A TOOL TO ASSESS THE POSSIBLE TOXIC EFFECTS OF PERSISTENT ORGANIC POLLUTANTS (POPs): A STUDY WITH SPECIAL REFERENCE TO THIAMINE DEFICIENCY, EROD INDUCTION AND THE BURSA OF FABRICIUS

Daphne Françoise de Roode

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Photo front page: 3 day old chicken embryo on yolk sac, Daphne de Roode

Ervaring is als een lamp op je rug –
het verlicht de weg die je reeds gegaan bent

Confucius (551-479 b.c.)

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

The primary aim of the study described in this thesis was to test the hypothesis that contaminant induced thiamine deficiency plays a role in avian embryotoxicity. A second aim was to assess the present contamination pressure on the common guillemot (*Uria aalge*) in the Baltic Sea, and the oystercatcher (*Haemopterus ostregalus*) in the Netherlands. Model compounds were used to test the hypothesis of thiamine depletion, and extracts of whole guillemots and of oystercatcher eggs were tested for their overall toxic effects in the chicken embryo. The chicken embryo was used as a sensitive model organism to detect potential toxic effects of the tested compounds.

Pollution and bird populations

Since the introduction of human made environmental contaminants (xenobiotics) in the environment, populations of birds have been shown to suffer from their adverse effects. Many studies have been reported in the fifties and sixties demonstrating mass mortalities of birds after various applications of pesticides, including chlorinated hydrocarbon compounds. During the first decade, the emphasis of the studies was on pesticides, but later it was recognized that other compounds, like metals and PCBs, compromised bird populations as well. Mortality was mainly attributed to pesticides. Especially the large-scale application of seed dressings caused acute mortality among species like pigeons, waders, ducks and geese, while delayed mortality occurred in many birds of prey, and population declines could be attributed mainly or at least partially to the increased death rate caused by the seed dressing chemicals (see review by Koeman, 1979). Turtle *et al.* (1963) demonstrated that dieldrin, aldrin and heptachlor in seed dressings were mainly responsible for observed deaths among wood pigeons and pheasants. Ratcliffe (1963) suggested that environmental change was responsible for the dramatic declines among bird populations, especially the peregrine falcon, in Great Britain. Subsequently, evidence accumulated showing the causative role of pesticides in population declines (for a review, see Moore, 1966). For instance, Prestt (1965) reported declines among British populations of sparrowhawk, kestrel, barn owl and tawny owl, which could be associated with toxic chemicals. Jefferies and Prestt (1966) and Prestt *et al.* (1968) found enhanced levels of toxic substances in dead peregrines, lanners and rough-legged buzzards, providing evidence for the causative role of these chemicals in population declines. In California, high mortality among fish-eating birds was caused by exposure to toxaphene, and DDT was suggested to play a role as well (J.O. Keith, 1966). Also in the Netherlands, evidence was obtained for the relation between accumulation of chlorinated hydrocarbons and increased mortality among several bird species (Koeman and van Genderen, 1966; Koeman, 1971; Koeman *et al.*, 1972a, 1972b and 1973). Strong evidence was provided for the causative role of pesticides in mortality cases among birds in the Netherlands (van Lieshout and Hoskam, 1972).

In the sixties it became clear that not only pesticides were responsible for the effects observed, but that other chlorinated industrial compounds, especially PCBs, played a role as well (Jensen, 1966). In accordance, decreases in reproductive success of bird species all over the world were related to enhanced levels of organochlorine contaminants in their eggs, chicks or adult bird tissues (Ames, 1966; J.A. Keith, 1966; Jefferies, 1971; Blus *et al.*, 1974; Wiemeyer *et al.*, 1975; King *et al.*, 1977; Noble and Elliott, 1990; Bosveld and van den Berg, 1994; Murk *et al.*, 1994; van den Berg *et al.*, 1994). In all these cases, persistent organochlorine compounds, such as PCBs, dioxins, dieldrin and DDE, were found to be the major contaminants present. Due to their lipophilic character, they tend to bioaccumulate in the lipid stores of organisms, and hence can reach appreciable concentrations.

A remarkable finding was the discovery of eggshell thinning in several birds of prey in Britain by Ratcliffe in 1967. It was shown that since 1945, eggshell thickness had decreased and had to be considered as an additional effect of organochlorines, especially DDT, affecting bird populations. Concurrently, raptorial and fish-eating birds in the United States were reported to suffer from catastrophic declines, associated with decreases in eggshell thickness (Hickey and Anderson, 1968). Evidence for the causative role of DDT in eggshell thinning was obtained from comparative studies on several bird species in which eggshell thickness was compared to eggshell thickness before 1945 (Ratcliffe, 1970; Anderson and Hickey, 1972; Douthwaite, 1992). Moreover, experimental evidence was produced for the dose related induction of eggshell thinning by DDT and DDE in a number of studies (Heath *et al.*, 1969; Bitman *et al.*, 1970; Peakall, 1970; Stickel and Rhodes, 1970; Wiemeyer and Porter, 1970; Jefferies, 1971; Lundholm, 1997). Eggshell thinning was also reported as one of the effects of DDE in a declining population of sparrow-hawks in the Netherlands (Koeman *et al.*, 1972c), as well as in cormorants, herons, sandwich terns and eider ducks (Koeman *et al.*, 1972b).

Another mechanism causing decreases in reproductive success is embryotoxicity. Embryotoxicity in birds has been associated with the presence of various organochlorine contaminants in eggs (Gilbertson *et al.*, 1976 and 1991; Nisbet and Reynolds, 1984; Elliott *et al.*, 1988; Tillit *et al.*, 1992; Becker *et al.*, 1993; Giesy *et al.*, 1994; Ludwig *et al.*, 1996; Bowerman *et al.*, 1998; Thyen *et al.*, 2000). Effects described include mortality, growth retardation, edema, deformities, liver enlargement, porphyria, liver necrosis and decreased immunocompetence. Evidence for the causative role of contaminants as obtained from laboratory studies is firm (Vos and Koeman, 1970; Carlson and Duby, 1973; Tumasonis *et al.*, 1973; Hoffman, 1979; Hoffman and Eastin, 1981; Gustafsson *et al.*, 1994; Summer *et al.*, 1996; Bosveld *et al.*, 2000). In addition, in many field situations, the circumstantial evidence for contaminantion as a responsible factor is convincing (as reviewed by Gilbertson *et al.*, 1991).

Recently, endocrine disruption was suggested to be a plausible explanation for avian reproductive problems (Feyk and Giesy, 1998; Bowerman *et al.*, 2000). However, even though feminization of male embryos was found after DDT exposure (Fry and Toone, 1981), the evidence for endocrine disruption in birds is limited (Janz and Bellward, 1996 and 1997; Bishop *et al.*, 1998; Vos *et al.*, 2000).

Decrease in pollution – consequences for bird populations

With the decreased release of contaminants during the last decades, bird populations have been observed to recover. Several authors report on the recovery of great blue herons in British Columbia (Sanderson *et al.*, 1994), peregrine falcons in Texas (Henny *et al.*, 1996), bald eagles from the Canadian Great Lakes (Bowerman *et al.*, 1998; Donaldson *et al.*, 1999) and New Jersey ospreys (Clark *et al.*, 2001). Also in the Netherlands, populations of sparrow-hawks, common kestrels, herring gulls, sandwich terns and common terns showed remarkable increases after the ban on organochlorines (Bijlsma *et al.*, 2001). However, Grasman *et al.* (1998) demonstrated that although fish-eating birds from the Great Lakes recovered on a regional scale, contaminants are still associated with reproductive and physiological effects.This was also observed for bald eagles in the Great Lakes (Bowerman *et al.*, 1998). In addition, although contaminant levels have declined and massive population declines belong to the past, pollution is still going on (Risebrough, 1986; Elliott *et al.*, 1992; Bignert *et al.*, 1998) and some bird populations still suffer from toxic effects (Grasman *et al.*, 1996; Jarman *et al.*, 1996; Custer *et al.*, 1999; McCarthy and Secord, 1999; Stansley and Roscoe, 1999). Recently, it was found that oystercatchers foraging at the Zeehavenkanaal area in the Netherlands are exposed to high levels of hexachlorobenzene (Eggens *et al.*, 2000); however, the toxic consequences of this exposure were not known. A study to reveal possible toxic effects in the embryos is described in this thesis. In addition, the present contaminant pressure in the Baltic common guillemot, which is still exposed to various pollutants, was studied.

Pollution and thiamine deficiency in the Baltic Sea area

One of the most heavily polluted sites in Europe is the Baltic Sea (Koistinen *et al.*, 1995). High concentrations of dioxins, dibenzofurans, PCBs and PCDEs have been measured in fish, seal and fish-eating birds (Koistinen, 1990; Koistinen *et al.*, 1995; Vuorinen *et al.*, 1997). Levels of polychlorodibenzo-*p*-dioxins, dibenzofurans and coplanar PCBs were shown to be the highest ever reported in wildlife (Tarhanen *et al.*, 1989). Despite a general decrease in contamination levels in the period from 1970 to 1984 in fish, fish-eating birds and seals, some compounds did not show a decreasing trend (Andersson *et al.*, 1988). Concentrations of PCBs and DDT in eggs of the common guillemot decreased in the period from 1969 to 1995, but this decrease had leveled off from 1985 and elevated levels of contaminants were still detected in 1995 (Bignert *et al.*, 1995 and 1998; Odsjö *et al.*, 1997).

Fish species living in the Baltic Sea and the Great Lakes show enhanced mortality during the early life stages. In the Baltic Sea, this was first observed in 1974, and, as it was supposed to be the consequence of environmental pollution, the syndrome was designated as M74 (Milieu 1974: Norrgren *et al.*, 1993). In the Great Lakes, the syndrome is called EMS, or Early Mortality Syndrome (Honeyfield *et al.*, 1998a). These syndromes are highly comparable and have caused massive mortalities among salmon and trout species since the early 1990s (Bengtsson and Hill, 1998; Honeyfield *et al.*, 1998a). One of the most striking similarities between the syndromes is that all eggs that produce offspring dying of the syndrome contain low levels of thiamine (Brown *et al.*, 1998a; Fitzsimons *et al.*, 1998; Honeyfield *et al.*, 1998a; Hornung *et al.*, 1998). Treatment of affected fry with thiamine prevents the fry from dying (Amcoff *et al.*, 1998): in fact, a reversal from 100% mortality to 90-100% survival was achieved after this treatment (Bylund and Lerche, 1995; Amcoff *et al.*, 1998). As

high contaminant levels were measured in fish producing affected offspring, these were suggested to play a role in the mechanism causing the mortality syndromes (Vuorinen *et al.*, 1997; Åkerman and Balk, 1998). In addition, hepatic cytochrome P450 enzymes were induced in both yolk sac fry suffering from M74 and adult feral females producing offspring affected by M74 (Norrgren *et al.*, 1993). Thus, it was hypothesised that contaminants induced thiamine depletion in fish suffering from M74 or EMS (Norrgren *et al.*, 1993; Balk *et al.*, 1998; Åkerman *et al.*, 1998; Pesonen *et al.*, 1999). If this hypothesis is correct, other organisms exposed to the same contaminants as the salmon and trout would be expected to display depletion of thiamine as well. Because of their exclusive fish-eating habits, fish-eating birds accumulate substantial levels of lipophilic organic pollutants. The common guillemot (*Uria aalge*) is a sedentary fish-eating bird species, and has been proven suitable for monitoring (Stolt *et al.*, 1991; Furness, 1993; Lyngs and Kampp, 1996; Joiris *et al.*, 1997). Contaminant levels in eggs of the common guillemot in the Baltic Sea were as high as 300 mg/kg lipids PCBs and over 600 mg/kg DDT, DDE and DDD in 1969; these levels declined to about 30% in 1982 (Bignert *et al.*, 1995). A further decline was reported for 1995, but the decrease had leveled off from 1985 and ongoing pollution with PCBs was suggested to be responsible for this observation (Odsjö *et al.*, 1997; Bignert *et al.*, 1998). Therefore, the common guillemot was chosen as the species to investigate if contaminants compromise bird populations at the current contamination levels in the Baltic Sea, and in addition, to assess the role of pollutant induced thiamine depletion in embryotoxicity.

Risk assessment studies using bioassays and biomarkers

In order to study the potential toxic effects of contaminants on bird embryos, the use of the chicken embryo assay has proven a valuable tool. An early description of an egg injection bioassay was given by McLaughlin *et al.* (1963). This assay employed the injection of chemicals, dissolved in various carrier solvents, into the fertilized chicken egg. This study was followed by many others. It was recognized that, in order to mimic the field situation, the delivery of a compound should be performed before onset of incubation and into the yolk (Koeman *et al.*, 1967; Walker *et al.*, 1967; Powell *et al.*, 1996; Henshel *et al.*, 1997). The chicken embryo is widely used as a model organism as it is known to be very sensitive towards the toxic action of chemicals (Brunström and Reutergård, 1986; Brunström, 1988; Brunström and Lund, 1988; Brunström, 1989; Brunström *et al.*, 1990a; Engwall *et al.*, 1994; Bosveld, 1995; Sanderson and Bellward, 1995). In addition, its development is well documented.
After the injection of a chemical into the fertilized egg, toxic effects in the developing embryo can be assessed by the use of biomarkers. A biomarker is defined as a biochemical, physiological, or histological indicator of either exposure to, or effects of, xenobiotic chemicals at the suborganismal or organismal level (Huggett *et al.*, 1992). This implies that different toxic endpoints can be assessed by using several biomarkers. A short description of the biomarkers used in the present study will be given below.

General toxic effects (nonspecific)

Mortality is the ultimate biomarker of toxic effects, in that it indicates the lethal action of a compound. Abnormalities among embryos can be used to indicate the teratogenic potency of compounds (Hoffman, 1979; Hoffman and Gay, 1981; Brunström, 1986; Walters *et al.*, 1987; Cho and Lee, 1990; Heinrich-Hirsch *et al.*, 1990), and measures

of embryo, yolk sac or organ weights indicate if compounds interfere with growth (Mayer *et al.*, 1992).

Induction of cytochrome P450
Induction of cytochrome P450 enzymes indicate the presence of contaminants that bind to the Ah receptor (Mason *et al.*, 1985 and 1986; Safe, 1990). Contaminants with the capacity to bind to this receptor usually have a coplanar, dioxin like figuration. Ethoxyresorufin-*O*-deethylase, or EROD, is an enzyme that belongs to the cytochrome P450 family (Rifkind *et al.*, 1994) and can be induced by coplanar PCBs and dioxins. Induction of this enzyme has been related to several embryotoxic endpoints in birds with consequences for reproductive output (Hoffman *et al.*, 1987 and 1993; Murk *et al.*, 1994; van den Berg *et al.*, 1994; Sanderson *et al.*, 1994). Therefore, an induction of this enzyme indicates increased exposure to organochlorines, and in addition, it may indicate the odds for a population to be compromised by the adverse effects of these contaminants.

Hepatic porphyrin accumulation
Porphyrins are precursors for heme proteins (Marks, 1985). Accumulation of hepatic porphyrins, also annotated as porphyria, in birds has been associated with the presence of polyhalogenated hydrocarbons, and indicates that the heme biosynthetic pathway is disturbed (Vos and Koeman, 1970; Vos *et al.*, 1971; Miranda *et al.*, 1987; Fox *et al.*, 1998; Kennedy and Fox, 1990). This may eventually lead to anemia or disruption of metabolic reactions. Porphyria has been suggested to result from the induction of aminolevulinic acid synthetase, the regulatory enzyme in the heme biosynthetic pathway, and decreased activity of uroporphyrinogen decarboxylase (Carpenter *et al.*, 1985a and 1985b; Miranda *et al.*, 1987).

Thiamine related enzymes
Transketolase and α-ketoglutarate dehyrogenase are enzymes that are directly dependent on thiamine diphosphate as a cofactor (Gibson *et al.*, 1984; Giguère and Butterworth, 1987; Schenk *et al.*, 1998; Olkowski and Classen, 1999). This cofactor is formed from thiamine and ATP by the enzyme thiamine-pyrophosphotransferase (Basilico *et al.*, 1979). Transketolase is situated in the pentose phosphate pathway, while α-ketoglutarate dehyrogenase plays an important role in the citric acid cycle (Stryer, 1988). Decreased activity of these enzymes has been associated with thiamine deficiency (Gibson *et al.*, 1984; Butterworth *et al.*, 1986; Giguère and Butterworth, 1987; Masumoto *et al.*, 1987; Balk *et al.*, 1998), concurrent with symptoms that resemble those described for contaminant exposed bird embryos, including leg weakness and vomiting, anorexia, ataxia, paralysis, hydropic degeneration of myocardium, retarded growth, nervous derangement and death (Swank, 1940; Shaw and Phillips, 1945; Charles *et al.*, 1972; Gries and Scott, 1972; Classen *et al.*, 1992). Transketolase is the most sensitive biomarker for thiamine deficiency in brain (Gibson *et al.*, 1984; Giguère and Butterworth, 1987; Remus and Firman, 1990; Itokawa, 1995; Balk *et al.*, 1998), whereas α-ketoglutarate dehydrogenase was found to be the best indicator of thiamine deficiency in the heart (Olkowski and Classen, 1999).
Contaminant induced thiamine depletion has been hypothesised to be the result of induced metabolism (Åkerman *et al.*, 1998; Balk *et al.*, 1998). This enhanced state of metabolism leads to an increased demand for NADPH and hence an increased ratio of NADP to NADPH, which upregulates the activity of glucose-6-phosphate

dehydrogenase in the pentose phosphate pathway. Therefore, an increased activity of this enzyme may be an indication of possible thiamine depleting processes, even before transketolase and α-ketoglutarate dehydrogenase activity decrease.

Acetylcholinesterase

Acetylcholinesterase is an important enzyme in neurotransmission, and its inhibition has been described in birds exposed to organophosphorous or carbamate insecticides (Busby *et al.*, 1991; Matz *et al.*, 1998; Parsons *et al.*, 2000). Inhibition of the enzyme in birds has been related to changes in behaviour (Busby *et al.*, 1991; Hart, 1993; Matz *et al.*, 1998; Bishop *et al.*, 2000; Parsons *et al.*, 2000) and abnormalities and mortality in developing embryos (Cho and Lee, 1990; Kaltner *et al.*, 1993).

Immune organs

The bursa of Fabricius is the organ in which B lymphocytes mature in the embryo and early chick (Glick, 1983; Nikolaidis *et al.*, 1988a). Decreased weight of this organ has been observed in wild birds exposed to dioxins and PCBs (Powell *et al.*, 1998; Bosveld *et al.*, 2000). Decreased lymphocyte density in the bursa of Fabricius appears to be a more sensitive biomarker and has been associated with exposure to PCBs (Nikolaidis *et al.*, 1988a and 1988b; Andersson *et al.*, 1991; Hoffman *et al.*, 1996; Grasman and Whitacre, 2001), extracts of particulate matter (Brunström *et al.*, 1992), Prudhoe Bay Crude Oil (Lusimbo and Leighton, 1996), and several pesticides (Misra and Bloom, 1991; Arias, 1995; Day *et al.*, 1995). In fact, B lymphocytes have been shown to be more sensitive than T lymphocytes (Nikolaidis *et al.*, 1988a and 1988b; Misra and Bloom, 1991; Wilmer *et al.*, 1992; Fox and Grasman, 1999). Finally, immunotoxic effects may decrease survival chances of the newly hatched chick and thus compromise reproductive success of the population (Grasman *et al.*, 1996; Smits and Bortolotti, 2001).

General outline of the experimental approach

In order to meet the aims of the project, several experiments were conducted using the chicken embryo as a model species. These experiments are described in *chapters 2-7* and in the *appendix*.

The first part of the thesis (*chapters* 2-4) describes the development of a bioassay to study effects of contaminants on thiamine dependent enzymes. *Chapter 2* describes the adaptation of existing injection bioassays for the use in studies with a focus on effects of contaminants on thiamine dependent enzymes. This bioassay is chemically validated in *chapter 3*, which describes the uptake of PCBs from the yolk by the developing embryo. Effects of some selected contaminants on transketolase activity *in ovo*, using the adapted bioassay, as well as *in vitro* are described in *chapter 4*.

The second part of the thesis describes two case studies to evaluate the risks of contaminants in wild bird species living in contaminated areas, using the developed bioassay. In *chapter 5*, extracts of whole guillemots from the Baltic Sea and the Atlantic Ocean were tested in the bioassay for their general toxic potency, as well as to assess the role of thiamine depletion in embryotoxicity, in order to assess the current contamination pressure on the Baltic guillemot. *Chapter 6* describes the use of the bioassay to estimate the risk for oystercatchers from a polluted site in the Netherlands, using extracts of collected eggs. In both studies, an induction of EROD activity is reported. In many field studies, EROD induction has been shown to be one of the most sensitive biomarkers, but evidence for its predictive value for adverse effects in bird populations is limited. In *chapter 7*, EROD activity is related to toxic

responses in order to estimate the predictive value of EROD induction for toxicity in birds. EROD induction throughout embryonal development is described in the *appendix*. In *chapter 8*, the major results are summarized and the overall conclusions that can be drawn from the research are presented.

CHAPTER 2

DEVELOPMENT OF A BIOASSAY TO TEST THE POSSIBLE ROLE OF THIAMINE DISTURBANCE AS A MECHANISM BEHIND POLLUTION-INDUCED REPRODUCTIVE FAILURES IN BIRDS

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Abstract
A test system was developed to examine the effects of environmental contaminants on thiamine homeostasis in bird embryos. This system employs fresh chicken egg yolk lipids as a vehicle for use in egg injection studies. Furazolidone, an antibiotic that is suspected to interfere with thiamine metabolism, was used as a positive control to evaluate the utility of the test system. It was determined that fresh chicken egg yolk lipids were preferable over chemical vehicles as it resulted in lower mortality rates (16% versus 23-62%) and did not induce any observable effects in the embryo. Injection of 1 mg/egg of furazolidone at day 0 of development resulted in decreased respiration followed by death, with mortality rates being twice as high as in carrier controls. In addition, transketolase activity, which was measured as an indicator of thiamine availability in the body, was decreased 25% in brains of 19 days old embryos. This mechanism may be of importance for effects of environmental contaminants in wild bird populations.

Introduction
Over the past decades, much effort has been put in the monitoring of ecosystem health after environmental contamination, for instance by studying bird populations. Birds at the top of the food chain have been considered to be key organisms for such monitoring programs, because they accumulate relatively high levels of persistent organic contaminants (POPs). Decreases in population densities were found to correlate with increasing levels of POPs. For some chemicals, like DDT, evidence was obtained for a causative relationship between concentration of the compound and reproductive failure. For other chemicals, such as PCBs, the evidence was less clear (Bowerman *et al.*, 1998, Custer *et al.*, 1999). However, for these compounds interactions were observed with the Ah receptor and metabolism of hormones which may have consequenses for reproduction (Bosveld *et al.*, 1994, Janz *et al.*, 1997). In addition to these, others may play a role as well. Even though some populations have been reported to be recovering, it is still important to obtain appropriate insight in the mechanism that might have been responsible for population declines. This may help to get a more complete understanding of structure relationships, which can be used to prevent undesirable effects of chemicals. A possible mechanism that has not been considered in birds, might have caused reproductive disturbances through the interaction with thiamine metabolism. This essential vitamin may have played a role in poor reproductive performance in fish from both the Baltic Sea and the Great Lakes in North America (Fitzsimons, 1995; McDonald *et al.*, 1998; Åkerman *et al.*, 1998; Fitzsimons *et al.*, 1999). Presently, this hypothesis is under investigation by some groups. The present study deals with a possible assessment of the mechanism in birds. In its phosphorylated form, thiamine acts as a cofactor for three essential enzymes in intermediary metabolism: transketolase (pentose phosphate pathway), pyruvate dehydrogenase (glycolysis) and α-ketoglutarate dehydrogenase (citric acid cycle). The reactions catalysed by these enzymes lead to the formation of chemical energy and intermediates for assimilation (Stryer, 1988). In rats, direct evidence has been provided for the interference of PCBs and DDT with thiamine metabolism after dietary exposure to these contaminants, which resulted in lowered thiamine levels in liver, blood and sciatic nerve, as well as decreased transketolase activity (Innami *et al.*, 1977; Yagi *et al.*, 1979). In fish, exposure to certain POPs has been suggested to reduce the available levels of the phosphorylated form of thiamine due to enhanced use of the cofactor, as a result of increased metabolism through the pentose phosphate

pathway. The increased metabolism is suggested to be driven by an increased use of NADPH through redox cycling (Åkerman *et al*,1998; Balk *et al.*, 1998). Other ways by which contaminants may induce symptoms of thiamine deficiency may be the binding of the compound with the active site of thiamine dependent enzymes, thus inactivating them as a catalytical site, or the inhibition of the phosphorylation of thiamine.
Severe disturbances of intermediary metabolism are suggested to afflict the general functioning of the individual, expressed as e.g. altered respiration activity and/or retarded growth. Avian test systems to evaluate embryotoxic effects have been developed (Brunström *et al,*. 1982, Henshel *et al.*, 1997, Powell *et al.*, 1996a). The use of an appropriate solvent is shown to be a critical factor in assessing effects of compounds. In an effort to eliminate adverse effects of the vehicle, the use of egg yolk lipids as a carrier was tested. Using this system effects on thiamine homeostasis were studied. Furazolidone was used as a model compound. This chemical is given as an antibiotic to poultry and has been associated with changes in thiamine status after therapeutic use (Ali and Bartlet, 1982; Ali, 1983). Typical symptoms and biomarker responses in birds exposed to furazolidone are anorexia and loss of body weight concomitantly with increased levels of lactate and pyruvate in blood, and increased activation of transketolase activity by thiamine diphosphate *in vitro*. However, the mechanism behind these observations is still unknown. Effects of furazolidone on the thiamine status in embryos have not been studied yet. In this study, the compound was injected at day 0 of development. End parameters investigated include respiration, enzyme activities, and mortality.

Materials and Methods
Fertile Lohmann brown chicken eggs were kindly provided by a local hatchery (Verbeek poultry farm, Lunteren). Propylene glycol (99%) was purchased from Boom, the Netherlands. Triolein (65%), tris-HCl, $MgCl_2.6H_2O$, sucrose, NaOH and tetrasodiumdiphosphate were obtained from Merck. Furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone), triosephosphate isomerase, α-glycerophosphate dehydrogenase, xylulose-5-phosphate, ribose-5-phosphate, NADH, NADP, fluorescamine and bovine serum albumin (BSA) were from Sigma.

Exposure technique and preparation of an improved carrier
Fertile eggs were weighed and evenly distributed among the treatment groups. All eggs were turned with a quick movement of the wrist and left to lay horizontally 24 hours before injection to let the germ cells float free to the top of the yolk and avoid trauma during the injection procedure. The day of injection was denoted as day 0. The eggshell was wiped with a tissue with 70% ethanol, and a 2 mm. hole was drilled over the air chamber, not penetrating the membrane. Then, the eggshell was wiped with 70% ethanol again, and 50 µl of the carrier was injected into the yolk using an Hamilton glass syringe equiped with a 0.8x40 21GA1½ needle. Several experiments were performed to assess effects of injection on mortality rates. In these experiments, a total of 187 control eggs was never injected, and a total of 65 eggs was mock injected (the needle was put in the yolk, but nothing was injected). Three different carriers were tested: triolein (n=46), propylene glycol (n=105) and a 12,000 g yolk lipids supernatant (n=67), of which a subsample was further analysed beyond mortality rates (controls, n=17; triolein, n=9; propylene glycol, n=17 and yolk lipids, n=17). The yolk lipids were prepared from fresh egg yolks from eggs of the same

batch as those that would be injected. Yolk was centrifuged for 2 hours at 12,000g and 15°C in an ultracentrifuge (Beckman), using sterilised polycarbonate ultrabottles with polyphenylene oxide screw closures (Nalgene) and a 50,2 Ti rotor. This yielded the separation of yolk into a more or less solid pellet and a clear, liquid, supernatant. The supernatant was gently pipetted off and used for injection. All handling was performed under sterile conditions. After injection, the holes were covered with a self-adhesive label, and the eggs were placed in an incubator, under 37°C and 55-65% relative humidity.
On days 5, 8, 11 and 15, eggs were candled for viability. Non-fertile eggs were discarded from the fertile eggs and mortality rates were recorded. On day 19, eggs were opened and livers and brains were dissected from the embryo and kept in 1 ml of icecold 0.25 M sucrose buffer until homogenisation within 30 minutes (in the triolein group, no brains were dissected). Homogenisation was performed using a glass potter tube and teflon plunger (Braun, Germany) at 1200 rpm. The homogenate was centrifuged at 7,000 g for 12 minutes and the supernatant was frozen in liquid nitrogen and kept at -80°C until analysis.

Enzyme activity analysis
Transketolase activity was determined spectrophotometrically at 340 nm from the change in NADH in a coupled reaction assay (Tate and Nixon, 1987), in which the formation of seduheptulose-7-phosphate and glyceraldehyde-3-phosphate from xylulose-5-phosphate and ribose-5-phosphate is catalysed by transketolase. Glyceraldehyde-3-phosphate is then converted to dihydroxyacetone phosphate by triosephosphate isomerase. Finally, NADH is oxidized to NAD by α-glycerophosphate dehydrogenase in the transition of dihydroxyacetone phosphate into glycerol-3-phosphate. 10 µl of the liver or brain supernatant was incubated at 30°C for 10 minutes in 240 µl 115 mM sucrose solution (pH 7.6) that contained 100 mM tris-HCl, 1.2 mM $MgCl_2$, 0.8 mM xylulose-5-phosphate, 0.2 mM NADH, 2 units triosephosphate isomerase and 0.2 units α-glycerophosphate dehydrogenase. Then, background activity was measured during 5 minutes at 30°C in a multiwell platereader spectrophotometer (VersaMax, Molecular Devices Corporation). The reaction was started by adding 10 µl of 10 mM ribose-5-phosphate to the medium and NADH consumption was measured during 5 minutes. A preincubation of 10 minutes was found to be suitable and gave reproducible results, with a doubling of activity when the sample volume was doubled. Protein content in the supernatants was measured fluorimetrically against a BSA standard (Lorenzen and Kennedy, 1993). Therefore, 50µl of diluted homogenate was mixed in 100 µl 0.05 M tetrasodiumdiphosphate buffer (pH 8.0) and 50 µl NaOH (0.1 N), after which the background fluorescence was measured at 360/475 nm (excitation/emission). Then, 50 µl 1.08 mM fluorescamine was added and the fluorescence was measured again.

Effects of furazolidone
Fertile chicken eggs were weighed and evenly distributed among 2 treatment groups (n=15 per group). In addition, 15 non treated eggs were incubated to check for background mortality rates. The injection procedure was as described for the previous experiment, using yolk lipids as a carrier. 50 µl of yolk lipid with or without furazolidone (1 mg/egg) was injected into the yolk at day 0 of development. Eggs were incubated until day 19 of development. CO_2 production was measured daily from day 15 to day 19 by placing the eggs in glass chambers, through which filtered air (Crossland element 703 particle filter) was led with a flow of 0.8 L/min. The

chambers were connected via a WA-328 24 channel gashandling unit (Analytical Development Co. Ltd., UK) to an infra red gas analyser (IRGA 225-2B-SS). Equilibration time was 5 minutes. At day 19 eggs were opened and embryos were sacrificed by decapitation. Brain and liver were removed and processed for TK activity measurements as described above.

Statistics
Mortality rates in different groups were tested with a t-test after logit transformation of the data and Bonferroni correction of α. Differences in enzyme activity and respiration between the different treatment groups were tested using a one-way ANOVA followed by a multiple range test. The significance level was stated at 5%.

Results

Effect of carriers on mortality and thiamine dependent enzyme activities
Our novel method, using yolk lipids as a carrier, resulted in an overall mortality of 16 %, which was not significantly different from untreated embryos (table 1). Chemical carriers resulted in significantly higher mortality rates. The novel carrier was found not to affect transketolase activity in liver whereas both triolein and propylene glycol did. Transketolase activities in livers were significantly higher in propylene glycol and triolein treated embryos than in controls (figure 1). In brains, no differences were found in enzyme activities between control and treatments (figure 2). The specific activity of transketolase in brain was significantly higher than the activity in livers from control and yolk lipid treated embryos. Based on these findings yolk lipid was further used as a carrier to evaluate the effects of furazolidone in chicken embryos.

Effects of furazolidone
The mortality rate among yolk-lipid injected controls was comparable to that among untreated eggs (table 2). Furazolidone treatment resulted in a doubling of the mortality rate over the control value, which was significantly ($p<0.025$) higher. It is noteworthy that mortality occurred during the first four days of incubation in both control and non treated eggs, whereas in the furazolidone treated group, embryos died throughout the entire incubation period.
No differences were found for biometric parameters (table 3). Average respiration in the furazolidone treated group was generally lower than in controls. This effect was significant on day 16, but not on later days (figure 3).
Transketolase activity in brain was significantly lower in furazolidone treated embryos than in controls ($p<0.05$). In the liver, no significant effect was observed (figure 4).

Table 1. *Mortality rates in chicken eggs injected with 50 µl of various carriers into the yolk before incubation. For literature data, numbers in brackets are control values from the respective study. Asterix indicates significant difference from non treated eggs (none). Literature data were not included in the statistical analysis.*

Carrier	n	mortality (%)	Reference
None	187	7	this study
Mock-injection	65	12	this study
Yolk-lipids	67	16	this study
Triolein	46	62*	this study
	64 (64)	37.5 (7.8)	Powell *et al.*, 1997
	60 (60)	53.3 (30.0)	Powell *et al.*, 1997
	60 (60)	26.7 (28.3)	Powell *et al.*, 1997
Propylene glycol	105	23*	this study
	38 (30)	55 (7)	Gebhart and van Logten, 1968
Lecithin/peanut oil emulsion in water	60 (58)	51.7 (13.8)	Powell *et al.*, 1996a

Table 2. *Mortality rates among non treated eggs, controls and furazolidone exposed embryos. Asterix indicates significant difference as compared to control ($p<0.05$).*

Group	n	mortality (%)
Non treated eggs	11	18.2
Control (yolk lipids)	10	20.0
Furazolidone (1 mg/egg)	12	41.7*

Table 3. *Total embryo and organ weights on day 19 of embryonic development, data presented in gram ± standard error.*

Treatment	n	Embryo	Yolk sac	Liver	Brain
Yolk lipids	8	23±0.8	8.2±0.6	0.45±0.025	0.83±0.063
Furazolidone (1 mg/egg)	6	21±0.5	8.0±0.8	0.46±0.018	0.77±0.033

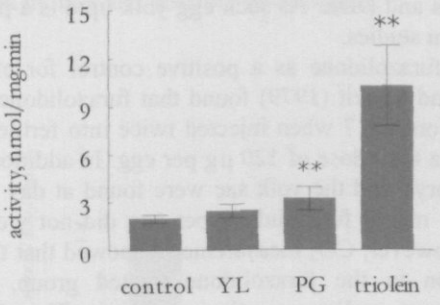

***Figure 1**. Transketolase activity in livers of 19 day old embryos. Values shown are means ± standard error over 13, 8, 6 and 4 individuals for controls, yolk lipids (YL), propylene glycol (PG) and triolein, respectively. **, significantly different from control ($p<0.01$).*

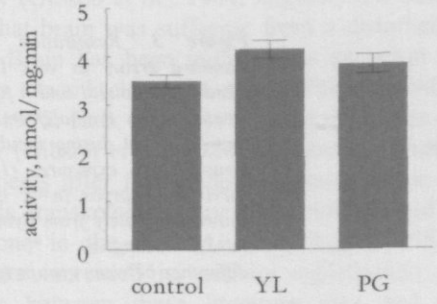

***Figure 2**. Transketolase activity in brains of 19 day old embryos. Values shown are means ± standard error over 13, 8 and 6 individuals for controls, yolk lipids (YL) and propylene glycol (PG), respectively.*

Discussion

In the present study, the development and evaluation of a test system using yolk lipids as a carrier for egg injection studies was described. It was found that yolk lipids did not enhance mortality, as chemical carriers did. Mortality rates caused by carriers in egg injection studies as reported by various authors range from 37.5 to 55% (table 1). In addition, it did not interfere with the thiamine dependent enzyme transketolase, which is an end parameter when studying the effects of contaminants on thiamine status. Moreover the novel carrier was found to have a good capacity to dissolve lipophilic compounds such as PCBs and DDE. As such egg yolk lipid is a promising solvent for use in avian egg injection studies.

The system was evaluated using furazolidone as a positive control for effects on thiamine homeostasis. Czarnecki and Sujarit (1979) found that furazolidone resulted in growth retardation, as observed on day 7 when injected twice into fertile chicken eggs on days 2 and 3, resulting in a total dose of 120 µg per egg. In addition, higher levels of glycogen in both the embryo and the yolk sac were found at day 5. In the present study, the application of 1 mg of furazolidone per egg did not yield direct evidence for growth retardation. However, CO_2 measurements showed that there was a decreased embryonic respiration in the furazolidone treated group, possibly indicating developmental impairment and/or growth retardation. The difference between groups was only significant at day 16, which can be explained by the time of death among the treated embryos, between day 16 and day 18. Thus, the difference was mostly due to the decreased respiration activity in embryos that died on day 17 or 18. It is hypothesised that the sensitive embryos, that would eventually show growth retardation, strongly influenced average body weight in the furazolidone treated embryos in the study performed by Czarnecki and Sujarit, but would have died in a later stage of embryonic development, as for example day 19 which was used to measure toxic endpoints in the present study.

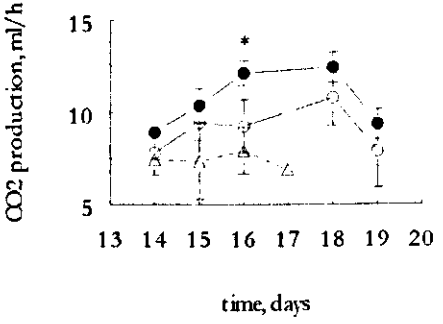

*Figure 3. Respiration (mean ± standard error) in yolk lipid (n=8; filled symbols) and furazolidone treated (open symbols) embryos from day 14 to 19 during incubation. For furazolidone exposure (1 mg/egg), surviving embryos (n=6; cirkels) are shown separately from dying embryos (n=3; triangles). *, significant difference between groups (p<0.05).*

The general decrease in respiration indicates that effects could be expected on the cellular and/or subcellular level. Intermediary metabolism yields both chemical energy and precursors for growth and further metabolism. A lowered transketolase activity, caused by a lowered thiamine diphosphate availability, strongly indicates that also the α-ketoglutarate dehydrogenase will be seriously affected (Butterworth *et al.*,

1986). This reduced activity gives in turn rise to a number of metabolic malfunctions, among which the first may be a temporary decrease in oxidative phosphorylation via the respiratory chain, resulting in a deficiency in energy (Aikawa *et al.*, 1984). The decrease in oxidative phosphorylation may be rather subtle, due to compensation mechanisms which result in an increased utilisation of certain amino acids by the citric acid cycle (Takahashi *et al.*, 1988). In the present study, a decrease in transketolase activity was observed in brain but not in liver. Thus, the observed decrease in CO_2 production may have been partly masked by a normal CO_2 production in liver and possibly other organs.

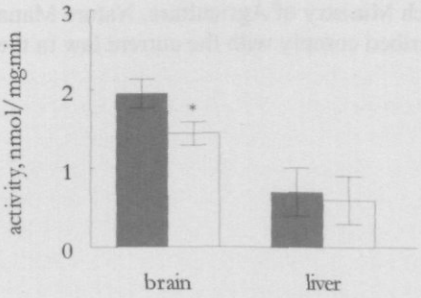

Figure 4. *Transketolase activity in brains and livers of 19 day old embryos. Values shown are means ± standard error of 8 and 6 embryos for yolk lipid (filled bars) and furazolidone (1 mg/egg; open bars) treatment, respectively. *, significantly different from control ($p<0.05$).*

The decrease in transketolase activity, being a sensitive marker for thiamine deficiency (Gibson *et al.*, 1984; Giguère and Butterworth, 1987; Balk *et al.*, 1998), showed that brain was suffering from a disturbed thiamine homeostasis while liver was not. Brain has been indicated as an organ that is very sensitive to thiamine deficiency (Swank, 1940; Shaw and Phillips, 1945; Giguère and Butterworth, 1987; Itokawa, 1995; Balk *et al.*, 1998); indeed in rats, it was found to be more sensitive than liver (Gibson *et al.*, 1984; Giguère and Butterworth, 1987). So far, enzymatic measurements after furazolidone exposure have only been done in adult birds. In blood cells, transketolase activity was not changed when birds were exposed to 0.04% furazolidone in the diet (Ali and Bartlet, 1982), but the addition of thiamine diphosphate increased its activity significantly more than in control birds. The difference between these literature data and the presently found decrease in transketolase activity may be due to the fact that the developing embryo requires more energy and precursors for biological macromolecules than the adult bird.

Other effects observed in furazolidone intoxicated birds include anorexia (Ali and Bartlet, 1982; Ali, 1983; Ullah *et al.*, 1998) and cardiomyopathy (Czarnecki and Jankus, 1975; Czarnecki and Grahn, 1980; Czarnecki *et al.*, 1982; Khan *et al.*, 1995), which cannot be measured easily *in ovo*, but are also suggested to be related to changes in intermediary metabolism.

In conclusion, the presented results indicate that yolk lipids are a suitable carrier to test effects of contaminants on thiamine homeostasis *in ovo*. In addition, it was shown that effects of furazolidone observed in this test system could be compared to those

found in adult birds. It should be noted however, that the implications will be completely different. Thiamine deficiency in adult birds can be reversed by the addition of extra thiamine to, or the withdrawal of the drug producing the deficiency from the diet (Charles *et al.*, 1972; Ali and Bartlet, 1982; Classen *et al.*, 1992; Olkowski and Classen, 1999), but the egg is a closed system in which such reversal is not possible. Therefore, if compounds interfere with thiamine status *in ovo*, this will have direct effects on embryo viability and thus on the reproductive performance of the population. Presently, a number of widespread environmental contaminants are being evaluated for their potency to interfere with the thiamine status in our *in ovo* test system.

Acknowledgements
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CHAPTER 3

EXPERIMENTAL UPTAKE OF PCBS FROM THE YOLK BY THE DEVELOPING CHICKEN EMBRYO

Accepted by Chemosphere

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Abstract
In this study, PCB uptake by the developing chicken embryo was measured after injection of two doses of Aroclor 1254 before incubation. It was shown that 2% of the injected PCBs was absorbed on day 13, and this increased exponentially to 18% at day 19. This exponential increase could be described by a similar model for both low and high injection doses. Differences in injection dose resulted in corresponding differences in concentration in the embryos. Lipid corrected concentrations in the embryo were stable through development from day 13 up to day 19 and could be predicted from injection doses by using a conversion factor of $0.15g^{-1}$.

Introduction
Effects of environmental pollution on birds have been described widely over the last few decades. For instance, embryotoxicity has been described for several compounds (Gilbertson and Fox, 1977; Hoffman, 1979; Gilbertson *et al.*, 1991; Giesy *et al.*, 1994; Grasman *et al.*, 1998; Kuiken *et al.*, 1999). In order to study the embryotoxic effects of contaminants, egg injection bioassays have been developed (McLaughlin *et al.*, 1963; Brunström, 1982; Powell *et al.*, 1996b; Henshel *et al.*, 1997). In a recently developed method, yolk lipids were chosen as a carrier as they did not interfere with mortality or enzymatic endpoints (chapter 2). However a further validation of that method was needed because an inhomogeneous distribution of the contaminant over the yolk may lead to differences in exposure of the embryo between eggs, but also in time. The fate of the contaminant after injection in the yolk is dependent on the initial behaviour of the carrier within the yolk, which is dictated by its density and physical properties (Walker, 1967).
In the present validation study, the uptake of PCBs was measured at several days during development. In addition, the relationship between injection dose and internal, lipid corrected concentration was assessed in order to assess whether nominal doses can be used to estimate exposure concentrations in the embryo. For this purpose, Aroclor 1254 was injected using yolk lipids (conform chapter 2), and PCBs were analysed at different time points during incubation. Time points were set at 13, 15, 17 and 19 days of development. At these days, it was expected that even in the low dosed group individual PCB congeners could be measured; day 19 was chosen as the final analysis day as this is the time point at which end parameters in exposure studies are normally measured (chapter 2).

Materials and Methods
Fertile Lohmann Brown chicken eggs were kindly provided by a local hatchery (Verbeek poultry farm, Lunteren). They complied to the dutch criteria for consumption eggs and therefore, the background concentrations of PCBs in the eggs were considered to be negligible. Aroclor 1254 was from Promochem.

Exposure technique
Eggs were weighed and randomly assigned to treatment groups; initial egg weights did not differ between groups so that differences in uptake of PCBs could not be attributed to differences in egg weight. Each group consisted of 60 eggs. Eggs were injected with 50 µl of solution on day 0 of incubation and incubated according to the method described in chapter 2. Exposure doses were 10 and 100 µg/egg of Aroclor. On days 13, 15, 17 and 19 of development, eggs were opened and embryos were

sacrificed by decapitation. They were stored at -20°C for chemical analysis. Embryos that died before day 19 were not analysed as it was not possible in all cases to estimate the exact time of death.

PCB analysis

PCBs were analysed according to van den Brink and Bosveld (in preparation; chapter 6). In short, samples of the carcasses were mixed with sodiumsulfate, and sohxlet-extracted with hexane. Individual PCB congeners were detected and quantified with capillary gas chromatography coupled with mass-spectrometry (GC-MS). Fat content of the carcasses was determined gravimetrically. Concentrations are expressed as µg/g lipid.

Statistics

Statistical analyses were performed under Genstat 5.5. Regression analyses using maximum likelihood were performed on the data to reveal trends in PCB concentration in time. Regression was considered significant at $p_{regr} \leq 0.05$. Differences between levels of PCBs in embryos in different groups but measured on the same developmental day, were detected using an ANOVA after log transformation of the data. The significance level was stated at 5%.

Results

On day 13 of development, PCBs were detectable in the body fat of the embryos at levels of 0.21 µg/embryo in the 10 µg/egg group and 2.1 µg/embryo in the 100 µg/egg group. When expressed as percentage of the injected amount, an accumulation of PCBs in the embryo was observed throughout development, reaching 18% in the low dose as well as the high dose group. Both groups showed similar uptake rates, which could be described by a single model, the best fit being an exponential increase ($p<0.001$, figure 1) including data for both groups.

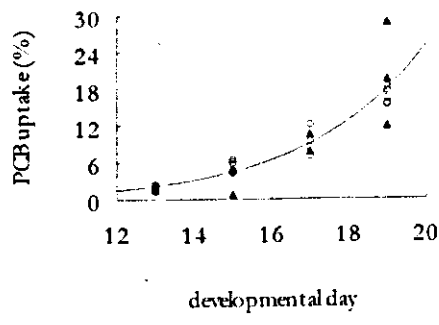

Figure 1. *Uptake of PCBs during development in embryos after injection of 10 µg/egg (open cirkels) or 100 µg/egg (filled triangles) into the yolk on day 0 of incubation, expressed as percentage of injected amount. The fitted model applies to both groups ($y=-0.22+0.0315*1.397^X$; $p<0.001$).*

PCB concentrations did not increase after day 13 (table 1; p=0.399 for the 10 µg/egg group and p=0.161 for the 100 µg/egg group). The average concentration over all days in the 10 µg/egg group was 1.4 µg/g lipid, and 16.4 µg/g lipid in the 100 µg/egg group. At all days, concentrations of PCBs were approximately 10 times higher in the 100 µg/egg injected group than in the 10 µg/egg injected group ($p<<0.05$, table 1). The ratio between concentrations (µg/g) and injected amount (µg) was 0.15 (g^{-1}) in both groups, which did not vary between exposure (p=0.123) or in time (p=0.758 and p=0.138 for the low and high dose group, respectively).

Table 1. Concentrations (µg/g lipid, average ± standard error) of PCBs in embryos at different developmental stages after injection of 10 or 100 µg Aroclor 1254 into the yolk at day 0 of incubation, numbers of measured embryos and ratio between measured concentrations in low dose and high dose group.

DD	Injection dose [a]				Ratio
	10 µg/egg	n	100 µg/egg	n	100 µg/egg : 10 µg/egg
13	1.3±0.48 [A]	4	14.9±1.5 [B]	4	11.5
15	1.34±0.27 [A]	6	13±3.1 [B]	2	9.7
17	1.6±0.48 [A]	5	21±6 [B]	2	13.1
19	1.5±0.31 [A]	5	17.5±2.1 [B]	3	11.7

DD, developmental day

[a] Values not sharing the same capital differ significantly from each other (ANOVA, $p<<0.05$)

Discussion

Several studies describe the gradual uptake of contaminants from the yolk. Koeman *et al.* (1967) demonstrated that dieldrin was distributed evenly over the yolk of chicken eggs within five days after injection, using propylene glycol as carrier. They also showed that the uptake of dieldrin from the yolk by the embryo proceeded upto at least 6 hours after hatching, after which the percentage of contaminant absorbed into the body was approximately 30% of the amount injected (calculated from their data assuming a whole animal weight of 30 grams). After injection of DDT into the yolk using dimethylsulfoxide as a carrier, 0.01-0.02% of the injected amount (based on a presumed embryo weight of 500 mg) was measured in five day old chicken embryos (Swartz and Schutzmann, 1981). For PCBs, no data regarding amounts of PCBs absorbed by the embryo after injection into the yolk are available from literature. However, some studies describe the distribution over the yolk sac and into the embryo. Brunström (1982) reported that PCBs were absorbed by embryos within 24 hours after injection of Aroclor 1248 on day four of development. A more detailed study indicated that PCB 49 injected into the yolk sac on day four of incubation, was spread homogeneously through the yolk on day 11 (Brunström *et al.*, 1982) and was present in the embryo within 24 hours after injection, but exact amounts were not presented. Recently, Bargar *et al.* (2001) described the gradual uptake of PCBs by the embryo from the yolk, after maternal deposition. Based on these results, a gradual uptake of PCBs from the yolk may be expected. In the present study, this gradual uptake was confirmed. The amount of absorbed PCBs, injected into the yolk on day 0 of incubation, in the embryo was shown to follow an exponential increase from 2.1% of the injected amount at day 13 of development up to 18% in both the 10 µg/egg and 100 µg/egg group, at day 19. Accordingly, Bosveld *et al.* (1995) reported PCB levels

in yolk sacs of common tern hatchlings which were fairly high, implying that some PCBs were still present in the yolk, and not yet absorbed by the embryo. In addition, the study by Bargard *et al.* (2001) shows a similar residue in the yolk sac and albumin after maternal deposition as in the present study. Thus, the fact that not all of the PCBs were absorbed by day 19 of development is not surprising, as the yolk is not yet completely absorbed by then.

The present study was designed to investigate the uptake of contaminants from the yolk in order to assess how injection doses relate to internal concentrations in the embryo. However, uptake may not be the only factor determining this actual concentration. Chicken embryos have been shown to be able to metabolize a tetrachlorobiphenyl and eliminate it via bile from day 11 of development (Brunström *et al.*, 1982). In another study, chicken embryos were found to metabolize PCBs and PAHs on day eight when exposed to these contaminants via injection into the air cell on day seven (Brunström, 1992). In effect, enzyme activities of the CYP450 family, thought to be capable of transforming PCBs, were measurable in whole embryo homogenates at day three of incubation, (Hamilton *et al.*,1983). The extent to which metabolism can affect measured levels of contaminants in embryos is illustrated by the disappearance of 94% of PAHs by day 18 of development, after injection into the yolk at day four of incubation, using a carrier composed of lecithin, peanut oil and water (Näf *et al.*, 1992). However, PCBs are in general more resilient to metabolic processes, so metabolism in birds is expected to be lower than for PAHs, constituting approximately a few percent (Bargar *et al.*, 2001; van den Brink and Bosveld, 2001). Hence, it may be expected that at day 19 only 20-25% of the total PCB load is actually absorbed by the embryo.

Lipid normalized concentrations in the chicken embryo were stable throughout development up to day 19. This implies that the uptake capacity of the embryo (the fat deposits) may be the limiting factor in the uptake of PCBs from the yolk. This is supported by the recent study by Bargar *et al.* (2001), in which a relation was found between lipid and PCB uptake by the embryo. In fact, the internal concentrations on lipid basis were a reflection of the exposure dose, showing a ratio of 10 between low and high dose injected eggs. The ratio between internal concentration and injected dose was similar in both groups at all days, allowing for the prediction of the actual concentration in the embryo at any developmental stage by multiplying the injected amount by a constant factor of 0.15 g^{-1}. It is sensed important that the factor that converts the injection dose into an internal, lipid corrected concentration, is independent on the dose applied. In this way, egg injection studies may be compared to field studies in which either initial concentrations or end concentrations are provided. In addition, as nominal concentrations were found to be related to internal embryonic concentrations in a linear way, this will facilitate mechanistic studies. In such studies, concentrations as well as toxicological parameters in exposed embryos are to be assessed; as it is rather difficult to measure both types of parameters in a single animal, this implies two animals are needed to give one data point. By using the conversion factor presented in this study, the use of test organisms can be reduced.

In conclusion, uptake of PCBs by the chicken embryo from the yolk throughout development can be described by an exponential curve, which is independent of the tested dose. Lipid corrected concentrations of PCBs in the embryo are stable through development from day 13 up to day 19 and can be predicted by multiplying the injection dose by a constant factor of 0.15 g^{-1}, both for low dose and high dose exposure.

Acknowledgements
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CHAPTER 4

EFFECTS OF FURAZOLIDONE, PCB77, PCB126, AROCLOR 1248, PARAQUAT AND *P,P'*-DDE ON TRANSKETOLASE ACTIVITY IN EMBRYONAL CHICKEN BRAIN

Accepted by Toxicology

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Abstract
The effect of *in ovo* exposure to PCBs, DDE and paraquat on transketolase activity was measured in 19 day old chicken embryos. Furazolidone was used as a positive control for decreased activity of the enzyme. The potency of contaminants to interact with transketolase was also tested in an *in vitro* system, using control brain 7,000 *g* supernatants containing the enzyme. No effects were found on transketolase activity after *in ovo* or *in vitro* exposure to PCB126, Aroclor, DDE or paraquat. PCB77 decreased transketolase activity *in vitro*, but only at concentrations that, extrapolated to *in ovo* exposure, would be lethal to the embryo. Furazolidone decreased transketolase activity both *in ovo* and *in vitro*. For this contaminant, thiamine residues were analysed in the yolk sacs, but no differences were found between exposed and non-exposed eggs. Transketolase is dependent on thiamine pyrophosphate as a cofactor, and therefore, the decreased enzyme activity could be the result of an interaction between furazolidone and thiamine metabolism. Since thiamine residues were not affected by furazolidone, and transketolase inhibition *in vitro* was similar to the inhibition after *in ovo* exposure, it was concluded that furazolidone interacted with transketolase on the enzymatic level rather than by a depletion of thiamine.

Introduction
The pentose phosphate pathway yields chemical energy (NADPH) and intermediates for assimilation (Stryer, 1988) and is as such crucial for the functioning of the organism. In this pathway, transketolase catalyses the transition of xylulose-5-phosphate and ribose-5-phosphate into seduheptulose-7-phosphate and glyceraldehyde-3-phosphate. These products can then be integrated into the glycolysis. Transketolase is dependent on thiamine pyrophosphate (Giguère and Butterworth, 1987; Schenk *et al.*, 1998), which is formed from thiamine and ATP by the enzyme thiamine-pyrophosphotransferase (Basilico *et al.*, 1979). Thiamine depletion has been suggested to be related to reproductive failures in salmon (Bylund and Lerche, 1995; Fitzsimons *et al.*, 1999). A toxicological factor was suggested to be involved in the mechanism causing thiamine depletion, as high levels of hepatic cytochrome P450 enzyme activity have been shown in fish that produced offspring suffering from thiamine deficiency (Norrgren *et al.*, 1993). It has been hypothesised that reproductive failures in birds occur via a similar mechanism (chapter 2), i.e. reproduction failures may result from contaminant-induced thiamine deficiency. Since transketolase is directly dependent on the availability of thiamine at the enzymatic level, decreasing enzyme activities are associated with reduced thiamine levels and therefore, transketolase activity was suggested as a biomarker for thiamine status in fish (Masumoto *et al.*, 1987; Amcoff *et al.*, 2000) and in developing bird embryos (chapter 2). In addition, contaminants may cause a reduction in transketolase activity by a molecular interaction with transketolase itself, thiamine pyrophosphate or thiamine-pyrophosphotransferase, which can be annotated as direct inhibition. In this case, transketolase cannot bind to thiamine pyrophosphate: the bioavailability of thiamine to transketolase is reduced. An example of a compound which has been suggested to affect transketolase in this manner is furazolidone (Ali and Bartlet, 1982). In this paper, this way of interaction will be annotated as 'interaction on the enzymatic level'. Indirect inhibition would be caused by the interaction between contaminants and intermediary metabolism, depleting thiamine levels as suggested for fish by Åkerman *et al.* (1998) and Balk *et al.* (1998). This would eventually lead to insufficient levels of thiamine pyrophosphate for transketolase to retain its activity.

Examples of such interactions may be contaminant induced CYP450 synthesis and activity, or redox cycling, which are both energy requiring processes and increase the demand for NADPH. In rats an interference of PCBs and DDT with thiamine metabolism has been described after exposure to these contaminants via their food. Thiamine levels were found to decrease in liver (Pélissier *et al.*, 1992), blood and sciatic nerve, and transketolase activity was lower than in control animals (Innami *et al.*, 1977; Yagi *et al.*, 1979). Paraquat is known to induce redox cycling in the cell (Grant *et al.*, 1980; Brown and Seither, 1983; Melchiorri *et al.*, 1998; Sanderson *et al.*, 1999), and has been shown to result in effects similar to those of thiamine deficiency in birds, including skeletal aberrations and reduced righting reflex (Swank, 1940; Shaw and Phillips, 1945; Smalley, 1973; Classen *et al.*, 1992). Based on the combined results it is hypothesised that the presence of high concentrations of PCBs, DDT and paraquat in bird eggs may affect the biochemical pathway in which thiamine and transketolase are involved in the production of metabolical energy, the pentose phosphate pathway.

Our present study aims to test this hypothesis, i.e. do contaminants influence the biological availability of thiamine, and, if the bioavailability is reduced, is this caused by a depletion of thiamine or by the direct interaction between contaminant and transketolase or its cofactor, thiamine pyrophosphate? The selected chemicals, furazolidone, PCB77, PCB126, Aroclor 1248, paraquat and p,p'-DDE, were tested *in ovo* and *in vitro*. In this way, it was possible to distinguish between thiamine depletion and interaction on the enzymatic level. Furazolidone was used as a positive control as it was found to decrease transketolase activity in bird embryos (chapter 2). The *in ovo* experiments were designed to expose embryos throughout development and then measure transketolase activity in the brains. For contaminants with capacity to induce cytochrome P450 activity, concentrations used were high enough to induce EROD activity (chapter 7 and Bosveld *et al.*, 1992), which, according to the hypothesis, could lead to a depletion of thiamine. As hypothesised, a decreased transketolase activity *in ovo* may result from either a depletion of thiamine or a direct interaction between contaminant and enzyme or cofactor. Therefore, for the compound that had an effect on transketolase *in ovo*, thiamine residue analysis was performed on yolk sacs, in order to establish the role of thiamine depletion in the observed decreased transketolase activity. In order to assess the capacity of the contaminants to interact directly with transketolase or its cofactor, *in vitro* experiments were conducted. This was also performed for the contaminants that did not decrease transketolase activity *in ovo*; as much higher concentrations could be used than in the *in ovo* experiments, this would give insight into the actual capacity of contaminants to interact with transketolase. These capacities may be apparent at contaminant concentrations close to lethal concentrations, and therefore hard to detect in *in ovo* experiments. The *in vitro* experiments consisted of exposure of control brain 7,000 *g* supernatants, containing transketolase and cofactors (available from *in ovo* experiments), to different concentrations of the test compounds, and measuring of transketolase activity.

Materials and Methods

Fertile Lohmann Brown chicken eggs were kindly provided by a local hatchery (Verbeek poultry farm, Lunteren). Propylene glycol (99%) was purchased from Boom, the Netherlands. Trichloric acid (TCA), ethylacetate, hexane, potassium hexacyanoferrate, tris-HCl, $MgCl_2.6H_2O$, sucrose, NaOH and tetrasodiumphosphate

were obtained from Merck. Dimethylformamide and acetonitrile were from Rathburn. Thiamine hydrochloride, thiamine monophosphate, thiamine diphosphate, paraquat (99%), DDE (99%), furazolidone (N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone), triosephosphate isomerase, α-glycerophosphate dehydrogenase, xylulose-5-phosphate, ribose-5-phosphate, NADH, NADPH, fluorescamine and bovine serum albumin (BSA) were from Sigma. PCB77 (purity 99.9%), PCB126 (purity 99.1%) and Aroclor 1248 were from Promochem.

In ovo exposure
Eggs were injected on day 0 of incubation and incubated according to the method described in chapter 2. Compounds were dissolved in yolk lipids and transketolase activity was measured in brain. Injected doses were 0, 0.00115, 0.0115, and 0.115 µg/egg for PCB126 (n=15); 0, 0.015, 0.15, 0.3 µg/egg for PCB77 (n=10); 0, 1, 10, 100, 1,000, 10,000 µg/egg for Aroclor 1248 (n=10); 0, 11.9, 119, and 1190 µg/egg for p,p'-DDE (n=15); 0, 3.3, 33, and 330 µg/egg for paraquat (n=15) and 0 and 1000 µg/egg for furazolidone (n=15; positive control; data reported in chapter 2). On days 12 and 15 of incubation, eggs were candled for viability and infertile eggs and dead embryos were discarded. After 19 days of incubation, eggs were opened and embryos were decapitated. Brains were dissected, frozen in liquid nitrogen and kept at -80° C for enzyme measurements. An extra experiment was performed injecting furazolidone (the compound that caused a decrease in transketolase activity) using filter sterilised propylene glycol as a carrier, resulting in exposure doses of 0, 10, 100 and 1,000 µg/egg, for thiamine residue analysis in yolk sacs.

In vitro experiment
7,000 *g* brain supernatants were incubated with contaminants in the transketolase assay as described below, in absence of ribose-5-phosphate and xylulose-5-phosphate. Concentrations ranged from 1nM to 1 µM and were increased stepwise by a factor 10. After 20 minutes, the substrates were added and activity was measured for 5 minutes.

Transketolase activity measurements
Homogenisation of brains was performed using a glass potter tube and teflon plunger (Braun, Germany) at 1,200 rpm. The homogenate was centrifuged at 7,000 *g* for 12 minutes after which the supernatant was frozen in liquid nitrogen and stored at -80°C until analysis. Transketolase activity was determined spectrophotometrically at 340 nm from the change in NADH in a coupled reaction assay as described by Tate and Nixon, 1987 and adapted for use in a multiwell platereader (chapter 2).

Protein determinations
Protein content in the supernatants was determined fluorimetrically against a BSA standard (Lorenzen and Kennedy, 1993).

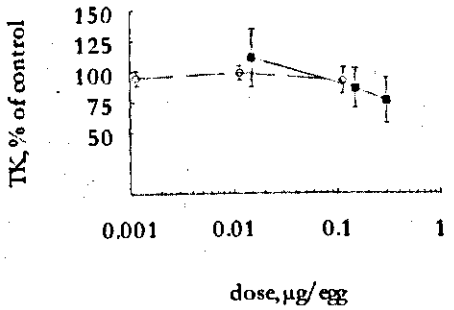
Thiamine analysis
Yolk sacs were freeze dried overnight and homogenised using morter and pistle. Thiamine and its phosphate ester thiamine pyrophosphate (TPP) were determined according to Brown *et al.* (1998b) with some modifications. Approximately 500 mg of sample was homogenised with 2.1 ml of ice cold 2% TCA. After boiling in a water bath and cooling in ice, homogenisation was repeated with 3.0 ml of ice cold 10% TCA. The homogenate was quantitatively transferred into a centrifuge tube and centrifuged at 14,000 *g*. The supernatant was washed four times with 8.8 ml of ethyl

acetate:hexane (3:2, v:v). Thiochromes were prepared and filtrated to HPLC. The HPLC apparatus consisted of two Waters 510 pumps, a Waters 717 Plus Autosampler equipped with a cooling unit, a thermostated column oven, a Waters Scanning Fluorescence HPLC Detector 474 and Millennium 32 software. The column was Hamilton PRP-1 (150 mm x 4.1 mm, particle size 5 µm). The injection volume was 20 µl and the total flow was 1.0 ml/min. The column temperature was adjusted to 35°C. The analysing time was 27 minutes using the gradient flow from eluents A to B:1 - 4 min 100% A, 4 - 14 min linear increase in eluent B to 100% and 14 - 27 min 100% A. Eluent A was 0.5% acetonitrile and 99.5% 25 mM phosphate buffer, pH 8.4 and eluent B 25% dimethylformamide and 75% 25 mM phosphate buffer, pH 8.4.
Blank samples were made by substituting NaOH with water. Standards were subjected to the same procedure as the tissue samples. With each sample set a sample from an egg homogenate was run as control. The recoveries of the determination were 82.8% for TPP and 106.1% for free thiamine, and the coefficients of variation from 39 determinations of the egg homogenate were 94.6% for TPP and 8.9% for free thiamine. High coefficients of variation for TPP are due to very low concentrations of this compound in the egg homogenate, its proportion of the total thiamine was less than 1%.

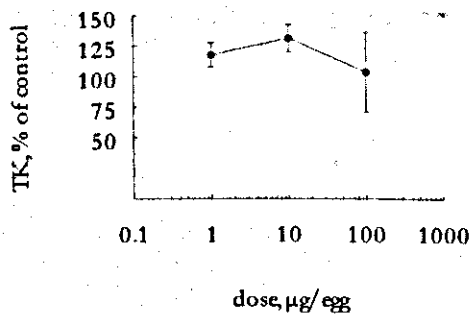
Statistics
Statistical analyses were performed under Genstat. Differences in enzyme activities and thiamine contents were tested using a one-way ANOVA followed by a multiple range test, as well as using regression analysis with exposure dose as description variable. Regression analysis of maximum likelihood was performed on the *in vitro* data in order to find a dose-response. Multiple regression analysis was performed on the thiamine data to find out which form(s) governed the activity transketolase. The significance level was stated at 5%.

Results

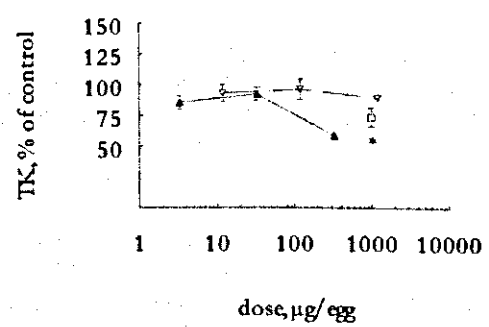
In ovo transketolase activity ranged from 3.5 to 7.5 nmol/mg.min in controls. In *ovo* exposure to furazolidone decreased transketolase activity in embryonal brain by approximately 27% (figure 1). None of the other tested contaminants affected transketolase activity in bird embryos significantly in a dose dependent way (figure 1). All compounds were tested *in vitro* as well to investigate if higher concentrations would affect transketolase activity by interaction on the enzymatic level rather than by depletion of thiamine. For furazolidone, the *in vitro* measurements revealed a significant dose dependent decrease in transketolase activity ($p<0.001$; table 1). PCB77 also decreased transketolase activity significantly; this was mainly due to the inhibition at the highest concentration ($p=0.024$; table 1). Aroclor, paraquat, p,p'-DDE and PCB126 did not influence transketolase activity at all ($p>0.05$; table 1).



Open cirkles: PCB126
Filled squares: PCB77



Aroclor



Open triangles: DDE
Filled triangles: paraquat
Open square: furazolidone

Figure 1. *Transketolase activity in brains of 19 day old embryos exposed in ovo to different doses of contaminants, expressed as % of control values. Values presented are means ± standard error. PCB126, n=11, 13 and 2; PCB77, n=5, 3 and 3; Aroclor, n=6, 7 and 4; DDE, n=10, 10 and 9, and paraquat, n=10, 5, and 1 for increasing doses, respectively, and furazolidone, n=12.*

***Table 1.** Transketolase activity in vitro in brain 7,000 g supernatants incubated with different contaminants. Values shown are means ± standard deviation. P-values indicate level of significance in difference as compared to control; n.d., not determined.*

nM	Fz	Paraquat	PCB126	Aroclor	*p,p'*-DDE	PCB77
0.001	n.d.	n.d.	n.d.	n.d.	n.d.	8.90±0.07
0.01	8.93±0.03	8.66±0.41	7.72±2.0	7.67±0.84	8.32±0.59	9.11±0.18
0.1	8.26±0.3	8.05±0.41	8.93±0.84	8.03±0.37	7.41±0.62	8.29±0.69
1	8.45±1.16	5.31±2.01	8.47±0.56	7.57±0.37	6.88±1.12	8.39±0.49
10	9.03±0.21	8.77±0.59	8.34±0.90	8.18±0.70	7.28±0.53	9.17±1.21
100	7.28±0.26 *p<0.001*	7.80±0.27	8.79±0.32	7.09±0.28	7.71±0.34	n.d.
1000	5.25±0.90 *p<0.001*	7.79±0.66	7.42±0.84	7.54±0.31	7.56±0.67	7.07±0.78 *p=0.024*

Furazolidone was the only compound for which an *in ovo* as well as an *in vitro* effect was observed, suggesting that the interaction was of enzymatic nature rather than one involving thiamine depletion. To validate this, thiamine levels were measured in yolk sacs. Thiamine pyrophosphate, free thiamine nor total thiamine levels were affected by furazolidone ($p>0.05$; table 2). Neither a relation was found between transketolase activity and thiamine residues in the yolk sac ($p>0.05$; figure 2).

***Table 2.** Thiamine residues in yolk sacs of 19 day old embryos exposed to different doses of furazolidone. TPP: thiamine pyrophopshate; free thiamine: thiamine without phosphate groups.*

µg/egg	TPP	free thiamine	total thiamine
0	0.44±0.29	1.38±1.49	2.23±1.89
10	0.47±0.25	1.07±0.91	1.96±1.15
100	0.54±0.38	1.23±0.39	2.14±0.48
1000	0.23±0.24	0.59±0.48	1.20±0.84

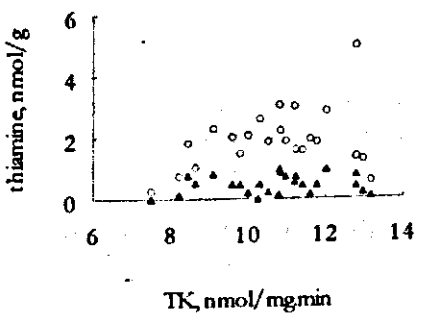


***Figure 2.** Relation between yolk sac residues of thiamine (thiamine pyrophosphate: filled triangles, and total thiamine: open cirkels) and transketolase activity (TK) in livers of 19 day old embryos exposed to different doses of furazolidone.*

Discussion

In this study, no effects on transketolase activity were found in embryos after *in ovo* exposure to PCB77, PCB126, Aroclor 1248, p,p'-DDE or paraquat. For these compounds, enzyme activities were always within the range of activities found for controls. *In vitro* data confirm that paraquat, p,p'-DDE, Aroclor and PCB126 do not interact with the enzyme, even at concentrations well above those expected in brain tissue after *in ovo* exposure. PCB77 showed a decrease in enzyme activity *in vitro*, but only at a concentration much higher than the applied dose in the *in ovo* experiment, assuming that the exposure concentration in the enzyme assay was comparable to 361 µg/embryo. This assumed exposure concentration in the embryo was calculated from the exposure concentration in the enzyme assay, which was added to 4 mg of brain, and thus could be expressed as pmol/mg brain. Assuming that this concentration resembles the concentration in the whole embryo, with an average embryo weighing 30 gram, the exposure dose in the embryo was calculated by multiplying the concentration by 30,000. With such high exposure doses *in ovo*, no survival can be expected; the LD50 after 3 days exposure starting at day 7 of incubation was found to be 0.5 µg/egg (Brunstrom and Andersson, 1988). Although PCB77 and PCB126 have a similar structure, a decrease in transketolase activity *in vitro* was not observed for PCB126. The reason for this difference is not clear, however, it should be noted that the observed decrease in transketolase activity for PCB77 was solely caused by the measurements at the highest concentration (statistical analysis excluding these three points yielded a value for p well above 0.05).

For furazolidone, the highest concentration used *in vitro* was comparable to the dose injected and resulted in approximately the same percentage of enzyme inhibition. This suggests that the effect of furazolidone on transketolase is the result of an interaction between furazolidone and either one of the enzymes or the cofactor involved. The assumption that furazolidone interacts with transketolase on an enzymatic level is confirmed by the results of the thiamine analysis. No effects were found on yolk sac residues of free thiamine, thiamine pyrophosphate or total thiamine, and there was no relation between yolk sac thiamine residues and transketolase activity. Although the relation between yolk sac residues and levels of thiamine in the cell is not exactly known, a decrease in yolk sac residues would be expected if transketolase activity was due to limited thiamine availability. Ali and Bartlet (1982) hypothesised that furazolidone inhibited thiamine-pyrophosphotransferase. However, no ATP is supplied to the system in the reaction assay, implying that the cofactor for transketolase (thiamine pyrophosphate) cannot be formed *de novo* during the reaction assay. Consequently, the inhibition of thiamine pyrophosphotransferase would not affect the availability of thiamine pyrophosphate in the *in vitro* test system. Therefore, the observed effects on transketolase activity *in vitro* is most likely due to a direct interaction between furazolidone and transketolase or thiamine pyrophosphate.

PCB126, Aroclor, DDE or paraquat did not affect transketolase activity *in vitro* or *in ovo*, whereas the observed effect of PCB77 on transketolase actvitiy *in vitro* may be deceptive, as this was only observed at a concentration where no embryo would survive. Thus, the suggested toxicological factor involved in the mechanism of thiamine deficiency in fish (Norrgren *et al.*, 1993), was not found to play a role in the developing chicken embryo as was tested in the present study.

Nevertheless, effects on transketolase activity have been described for mammals. PCBs and DDT decreased thiamine levels in mammals (Innami *et al.*, 1977; Yagi *et al.*, 1979; Pélissier *et al.*, 1992) as well as transketolase activity (Yagi *et al.*, 1979). If the mechanism behind these effects is based on an interaction between contaminants

and thiamine bioavailability, the discrepancy between mammals and bird embryos may be explained by the different circumstances the animals live in. Thiamine is an essential vitamin, implying it should be present in the daily diet of adult mammals. However, bird embryos live in a closed egg with thiamine supplies stored in the yolk sac (Adiga and Ramana Murty, 1983). When present at sufficiently high levels, this may prevent the embryo from contaminant induced thiamine deficiency and effects may only be observed when eggs receive reduced amounts of thiamine from the mother bird.

In conclusion, it was found that furazolidone inhibits transketolase activity, and that this effect as observed after *in ovo* exposure is due to an interaction between the compound and transketolase or its cofactor thiamine pyrophosphate. PCBs, paraquat and p,p'-DDE did not affect transketolase in the developing chicken embryo. It should be noted that up til now, no reports exist on the potency of other contaminants to interact with the pentose phosphate pathway in the developing bird embryo.

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CHAPTER 5

EFFECTS OF PERSISTENT ORGANIC POLLUTANTS PRESENT IN EXTRACTS OF GUILLEMOTS (*URIA AALGE*) FROM THE BALTIC SEA AND THE ATLANTIC OCEAN, ON THE DEVELOPING CHICKEN EMBRYO

Submitted to Environmental Toxicology and Chemistry

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Abstract
The Baltic Sea is a heavily polluted area. In order to assess the current contaminant pressure on the common guillemot (*Uria aalge*) living there, whole body extracts of guillemots from the Baltic Sea were prepared and subdivided over six fractions, which differed in composition due to lipophilicity and polarity of the contaminants. The fractions were tested in the chicken embryo assay and compared to fractions of Atlantic guillemot extracts. Fertilized chicken eggs were injected with 0.03, 0.3 or 3 bird egg equivalents (beq) of the contaminants present in the fractions and incubated for 19 days. End points were selected to cover several mechanisms that may play a role in reproductive failures in fish-eating birds. Fractions I and IV from the Baltic guillemots induced EROD activity up to 15 times in embryos exposed to 0.3 beq and up to 17 times in embryos exposed to 3 beq. Corresponding Atlantic fractions caused induction of EROD activity only at the higher dose of 3 beq. Morphological alterations were observed in the bursa of Fabricius of embryos exposed to the fractions that induced EROD, and for the Baltic fractions this was apparent at the dose of 0.3 beq. The higher toxic potency of fractions I and IV was confirmed by higher mortality and occurrence of malformations among embryos exposed to these fractions. No other effects were observed; morphometry, hepatic porphyrin levels, thiamine dependent enzymes and acetylcholinesterase activity were not affected by any fraction. Within the framework of interpretation of the results, a comparison was made between concentrations in the guillemot extracts and concentrations reported in field studies. In general, it was found that concentrations in the guillemot extract were lower than those associated with biomarker responses in other wild bird species. However, as the relative sensitivity of guillemot towards immunotoxic effects remains to be resolved, effects on their immunocompetence could not be excluded.

Introduction
The Baltic Sea is a heavily polluted area in which fish-eating birds, located at the top of the aquatic food chain, accumulate high levels of lipophilic persistent organic pollutants (POPs). Although there is no direct evidence that guillemots or related species are affected by these POPs, there are many data showing that high concentrations are stored in their bodies (Tarhanen *et al.*, 1989; Bignert *et al.*, 1995 and 1998; Koistinen *et al.*, 1995). The aim of the present study was to assess the toxic potential of the complex mixture of POPs present in the common guillemot using the chicken egg bioassay.
The common guillemot (*Uria aalge)* is a sedentary species at the top of the aquatic food chain and has therefore been used for the monitoring of polluted areas (Stolt *et al.*, 1991; Furness, 1993; Lyngs and Kampp, 1996). Eggs of guillemot were found to contain up to 300 mg/kg lipid PCBs and over 600 mg/kg lipid DDT, DDE and DDD in 1969; these levels declined to below 100 mg/kg lipid for both contaminant groups in 1989 (Bignert *et al.*, 1995). A further decrease to approximately 50 mg/kg lipid was reported for 1995, but the decrease had leveled off from 1993 and it was suggested that pollution with PCBs was still existent (Bignert *et al.*, 1998).
In order to investigate the potential pressure from contaminants on the common guillemot, birds from the Baltic Sea were extracted and the embryotoxic potency of the extracts was compared to that of extracts of birds living in the Atlantic Ocean, which was chosen as a reference site. Extracts of whole birds, without their head and feet, were prepared and divided into different fractions in order to separate classes of compounds with different physico-chemical characteristics. By applying this

approach, observed effects could be pinpointed to a defined group of substances. In addition, characteristics of unknown substances evoking responses might be elucidated as they would be fractionated to a certain class of compounds. Homogenates were extracted using either toluene or a mixture of hexane and acetone (50:50). Then, extracts were subdivided into three fractions, using hexane, hexane/dichloromethane (3:1), or acetone (Gustavsson *et al.*, 2001). These fractions were tested for their embryotoxicity in the chicken embryo assay as described chapter 2. End points measured were selected as biomarkers for different mechanisms that have been suggested to play a role in reproductive failures in fish-eating birds. These include EROD, transketolase, α-ketoglutarate dehydrogenase, glucose-6-phosphate dehydrogenase, acetylcholinesterase, hepatic porphyrins, histology of the bursa of Fabricius, morphometry and mortality.

EROD is a biomarker for contaminants that bind to the Ah receptor. An induction of EROD activity has been found to be correlated to abnormalities and low chick survival in wild birds (e.g. Hoffman *et al.*, 1987; Sanderson *et al.*, 1994; Murk *et al.*, 1996). Transketolase and α-ketoglutarate dehydrogenase are biomarkers for thiamine deficiency (Gibson *et al.*, 1984; Butterworth *et al.*, 1986; Giguère and Butterworth, 1987; Masumoto *et al.*, 1987; Balk *et al.*, 1998), which was suggested to play a role in embryotoxicity (chapter 2), and glucose-6-phosphate is an enzyme in the pentose phosphate pathway that is upregulated by an increased ratio of NADP to NADPH, which has been hypothesised to be the possible linking factor between contaminant induced metabolism and thiamine deficiency (Åkerman *et al.*, 1998; Balk *et al.*, 1998). Acetylcholinesterase is an important enzyme in neurotransmission, and its inhibition by organophosphorous and carbamate insecticides has been linked to changes in behaviour (Busby *et al.*, 1991; Hart, 1993; Matz *et al.*, 1998; Parsons *et al.*, 2000; Bishop *et al.*, 2000) and abnormalities and mortality in developing embryos (Cho and Lee, 1990; Kaltner *et al.*, 1993). Porphyrin accumulation can be induced by contaminants that also induce EROD activity, and is an indication of disturbed heme biosynthesis (Vos and Koeman, 1970; Vos *et al.*, 1971; Carpenter *et al.*, 1985a; Miranda *et al.*, 1987; Fox *et al.*, 1998; Kennedy and Fox, 1990). Eventually, a disturbance of the heme biosynthesis may lead to disfunctioning of hemoproteins such as hemoglobulin and cytochromes (Marks, 1985). As B lymphocytes have been reported to be more sensitive towards the toxic action of contaminants than are T lymphocytes (Harris *et al.*, 1976; Nikolaidis *et al.*, 1988a, 1988b and 1990; Brunström *et al.*, 1992; Wilmer *et al.*, 1992; Hoffman *et al.*, 1996), the bursa of Fabricius was chosen as the organ for studying effects on immune organs. Morphometry was measured in order to establish the influence of organ weights on eventual differences in other end points between groups, as well as to investigate the effects of the contaminants on growth and their teratogenic potency. Finally, mortality was monitored as an overall indication of toxicity.

Materials and Methods

Fertilised chicken eggs were kindly provided by Verbeek's Poultry farm, Lunteren. Guillemots were obtained as by-catch (accidentally taken in fish nets) from the Baltic Sea, outside the eastern coast of the Swedish island of Gotland in the Baltic proper (n=60) and from Røst Island, northern Norway, in the Atlantic Ocean (n=40).

Extracts

Extracts of guillemots from the Baltic Sea and Atlantic Ocean were prepared by Gustavsson *et al.* (2001). The extracts were fractionated into fractions, annotated as

fraction I-VI. First, two extracts of the homogenates were prepared, using either hexane/acetone (50:50) or toluene. The hexane/acetone extract was used to obtain fractions I-III, and the toluene extract yielded fractions IV-VI. Thus, the extracts were fractionated into three fractions each using hexane (fractions I and IV), hexane/dichloromethane (3:1) (fractions II and V) and acetone (fractions III and VI). Analysis of whole extracts of the birds was performed according to Koistinen *et al.* (1995).

Bioassays

In ovo exposure of chicken embryos was carried out according to the method described in chapter 2. Two experiments were performed, which were separated in time. First, fractions I, II and III were tested. In the second experiment, fractions IV, V and VI were tested. Fractions were evaporated to dryness under sterilized nitrogen, and yolk lipids (fractions I and II) or propylene glycol (all other fractions) were added. As acetone traces interacted with yolk lipids, it was not possible to inject the acetone fraction with this carrier; sterilised propylene glycol was used instead. In addition, at the time of the second experiment, some eggs were infected before onset of the experiment and therefore it was decided not to use yolk lipids, as these may spread an infection among eggs, but instead dissolve fractions IV-VI in propylene glycol. Although it has been described that yolk lipids are preferred as a carrier when thiamine dependent enzymes are to be studied in order to test the hypothesis of contaminant induced thiamine deficiency (chapter 2), it was decided to use propylene glycol in the second experiment in order to prevent the infection of large numbers of eggs. This carrier had only minor effects on transketolase (chapter 2), and in addition, no effects on thiamine dependent enzymes were observed in embryos exposed to fractions I and II, implying that no effects were to be expected in the second experiment.

The injection volume was always 50 µl, resulting in 0.03, 0.3 and 3 bird egg equivalent (beq) of contaminants per egg. These concentrations were chosen as they would represent the range of concentrations found in the field situation. Controls were injected with 50 µl of the corresponding carrier. Bird egg equivalents were calculated from the assumption that the body of one adult contained 10 times the amount of contaminants present in one egg (based on their difference in weight). Thus, 3 beq was 0.3 adult bird body burden. Injection was performed under sterile conditions on day 0 of incubation. Then, eggs were incubated for 19 days. Survival was recorded and dead and non fertile eggs were discarded. At day 19, embryos were weighed and decapitated. Yolk sacs were weighed; liver, heart and brain were dissected, weighed, frozen in liquid nitrogen and kept at -80°C until preparation. The bursa of Fabricius was dissected, weighed and fixed in formaline.

Enzyme assays

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in liver microsomes as described by Kennedy *et al.* (1993). In short, livers were homogenised in phosphate buffer (0.125 M, pH 7.6) containing EDTA (0.1 M). Subsamples of the homogenates were stored at -80°C for porphyrin analysis, and the rest was centrifuged at 15,000 *g* for 12 minutes at 4°C. The supernatants were centrifuged at 150,000 *g* for 72 minutes at 4°C, and the pellets were resuspended in phosphate buffer with 20% glycerol. The microsomes were stored at -80°C until analysis. For the EROD assay, 10 µl of microsomes were added to 220 µl buffer containing tris-HCl (80 mM, pH 8),

NaCl (80 mM), bovine serum albumine (1 mg/ml) and 7-ethoxyresorufin (1.1 µM). The reaction was started by the addition of 20 µl NADPH (3mM), and the production of resorufin was measured fluorimetrically over 15 minutes at wavelengths of 530/590nm excitation/emission, at 37°C.

For transketolase and glucose-6-phosphate dehydrogenase, brains were homogenised in phosphate buffer (pH 7.4, 0.1 M) and centrifuged at 10,000 *g* for 12 minutes. Supernatants were stored at -80°C until analysis.

Transketolase activity in brain supernatants was measured in an adapted assay based on Tate and Nixon (1987) as described in chapter 2. Glucose-6-phosphate dehydrogenase activity was measured spectrophotometrically in a multiwell plate from the production of NADPH at 340 nm. in 10,000 *g* brain supernatants. In short, 20 µl of supernatant was added to 200 µl buffer containing tris-HCl (55 mM), $MgCl_2$ (22 mM), sucrose (188 mM) and NADP (0.55 mM). The reaction was started by the addition of glucose-6-phosphate (4.5 mM), and the production of NADPH was measured for 5 minutes at 37°C and 340 nm.

α-Ketoglutarate dehydrogenase activity was measured in heart homogenates in a multiwell plate according to Olkowski and Classen (1999) with minor adaptations. In short, hearts were homogenised in buffer (pH 7.6) containing KH_2PO_4 (50 mM), EDTA (1 mM) and mercaptoethanol (2 mM). Homogenates were frozen at -80°C to lyse blood cells. Then, homogenates were thawed on ice and diluted in a phosphate buffer (dilution buffer; pH 7.6) containing tris-HCl (63 mM), $MgCl_2$ (1.25 mM), $CaCl_2$ (1.25 mM), dithiothreitol (0.63 mM), 2-mercaptoethanol (0.63 mM), EDTA (0.63 mM) and triton X100 (0.13%). 20 µl of the diluted sample was added to 200 µl dilution buffer with NAD (3.1 mM), coenzyme A (0.39 mM) and α-ketoglutarate (2 mM). Activity was measured at 340 nm and 37°C for 5 minutes.

Acetylcholinesterase activity was measured according to the assay described by Ellman *et al.*(1961).

All enzyme activities were calculated per mg protein present in the samples used for measurements. Protein contents were determined fluorimetrically at wavelengths of 360/460nm excitation/emission against a BSA standard according to Lorenzen and Kennedy (1993).

Hepatic porphyrins

Porphyrins were measured fluorimetrically in multiwell plates. Liver homogenates (from the liver microsomal preparation) were diluted in water and extracted using a buffer containing tris-HCl (18.75 mM, pH 8.0) and trichloroacetic acid (15.6%). Then, 180 µl of the extracts were added to 20 µl NaCl (0.9%), the plate was shaken and placed on a UV lamp for 5 minutes, after which it was centrifuged at 5,000 *g*. The supernatants were transferred to a new plate, which was measured at 400/590nm excitation/emission. Porphyrin levels were calculated in µg per g liver.

Histology

Bursae of Fabricius were fixed in formaline and dehydrated through a series of increasing ethanol concentrations. Finally, organs were embedded in paraffin and semithin sections (7µm) were sliced at a microtome. Slices were rehydrated and stained with eosine/haematoxiline, and enclosed using paramount. Slices were studied double blind under the microscope and appearance of the bursa of Fabricius was classified according to observed lymphocyte density. The appearance of follicles was graded along the axis of normal, with high lymphocyte density (class 1) to clearly abnormal, with decreased lymphocyte density and lighter staining (class 4), with two

intermediate classes, as depicted in figure 1. The average index was then calculated per group.

Statistics

Statistical analyses were performed under Genstat 5.5. Mortality rates, occurrence of malformations and histological index were tested with a general regression model after logit transformation of the data and under a binomial distribution. The occurrence of malformations and histological index were tested with a one-sided significance level of 10%, as the expected effect was a an increase in malformations and bursal index after exposure to the contaminants. Mortality was tested with α at 5%. ANOVA, with α at 5%, was used to detect differences between groups for organ weights, enzyme activities or porphyrin levels as compared to control values. Regression analysis of maximum likelyhood was performed on the data with log transformed doses to detect dose-response relationships; α was stated at 5%. Data for controls injected with yolk lipids or propylene glycol were tested againts each other and pooled when no differences existed.

Data on activities of transketolase, glucose-6-phosphate dehydrogenase, α-ketoglutarate dehydrogenase and acetylcholinesterase are expressed as % of the respective control value, but statistical analysis was performed on raw data.

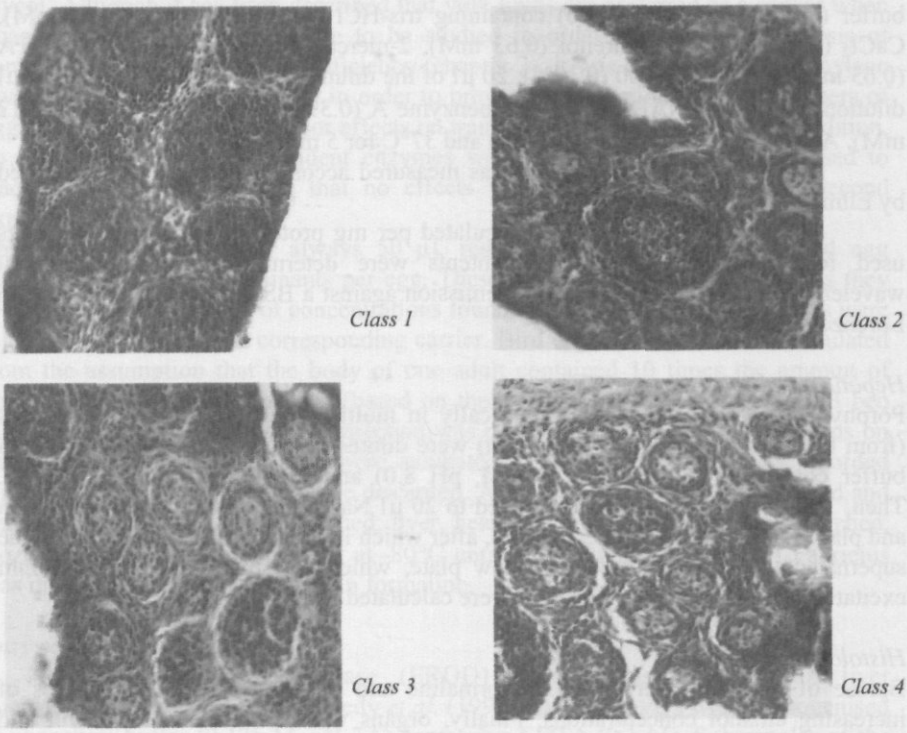


Figure 1. *Classification of bursa appearance. Bursa index ranges from normal (class 1) to clearly abnormal (class 4), with two intermediate classes. Photographs: magnification 20x, HE stain.*

Results

Chemical analysis

Table 1 lists the contaminants found in extracts of whole bodies of guillemots, as measured by Rantalainen et al. (2000). From this table, it is clear that the different extraction procedures yielded very similar extracts. Therefore, fractions I, II and III were expected to be relatively similar to fractions IV, V and VI, respectively. The same extraction and fractionation procedure was performed on salmon samples, and analysis of those fractions revealed that practically all of the known POPs with high lipophilicity and low polarity were distributed to fractions I and IV (Rantalainen, personal communication).These analyses further described that diphenyl ethers and polychlorinated dibenzofurans were detected in small parts (±2%) in fractions II and V, whereas planar chlorinated hydrocarbons (coplanar PCBs and chloronaphtalenes) resided slightly more in these fractions (up to 6%). Fractions III and VI contained traces of PCBs, DDE and polybrominated diphenyl ethers. Guillemot fractions were not tested, but a similar distribution over fractions is to be expected.

Table 1. *Contaminants found in hexane/acetone and toluene extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Concentrations are expressed per g lipid.*

Extract	Hexane/acetone		Toluene	
Contaminant group	Atlantic	Baltic	Atlantic	Baltic
2378TCDD, pg/g	11	95	6	62
Dioxins [a], pg/g	216	931	120	702
Furans [b], pg/g	3381	8397	1366	3275
PCBs [c], ng/g	819	7055	670	6798
OC pesticides [d], ng/g	1077	7211	747	7778
Coplanar PCBs, ng/g	10.5	79.4	6.7	66.5
Chlorinated napthalenes, ng/g	2.1	17.7	1.5	16.6
Total PCDE [e], pg/g	5349	17313	2460	9697
Total PBDE [f], ng/g	127	344	81	248

[a], polychlorinated dibenzo-p-dioxins; [b], dibenzofurans; [c], these PCBs do not include the coplanar PCBs listed in this table; [d], pesticides include hexachlorocyclohexane, hexachlorobenzene, chlordane, DDE; [e], polychlorinated diphenylethers; [f], polybrominated diphenylethers

Bioassays

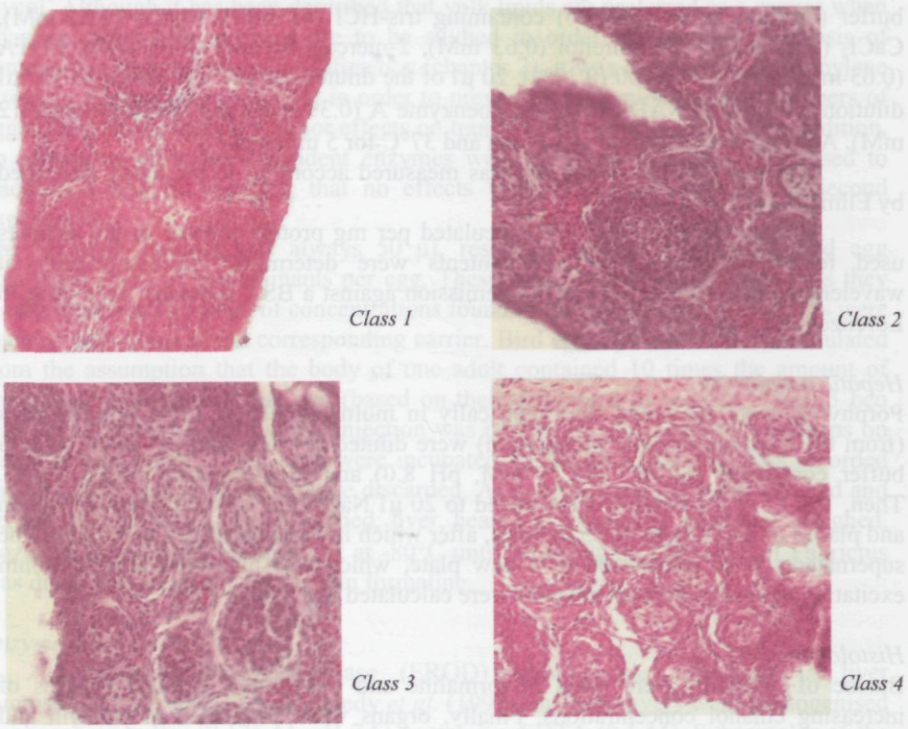
Mortality was not increased by treatment in the first experiment, which is probably due to high background mortality. In the second experiment, dose-dependent mortality increases were observed with fraction IV of the Baltic extract and with fraction V of the Atlantic extract ($p<0.05$, table 2).

intermediate classes, as depicted in figure 1. The average index was then calculated per group.

Statistics

Statistical analyses were performed under Genstat 5.5. Mortality rates, occurrence of malformations and histological index were tested with a general regression model after logit transformation of the data and under a binomial distribution. The occurrence of malformations and histological index were tested with a one-sided significance level of 10%, as the expected effect was a an increase in malformations and bursal index after exposure to the contaminants. Mortality was tested with α at 5%. ANOVA, with α at 5%, was used to detect differences between groups for organ weights, enzyme activities or porphyrin levels as compared to control values. Regression analysis of maximum likelyhood was performed on the data with log transformed doses to detect dose-response relationships; α was stated at 5%. Data for controls injected with yolk lipids or propylene glycol were tested againts each other and pooled when no differences existed.

Data on activities of transketolase, glucose-6-phosphate dehydrogenase, α-ketoglutarate dehydrogenase and acetylcholinesterase are expressed as % of the respective control value, but statistical analysis was performed on raw data.



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Results

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Table 1. *Contaminants found in hexane/acetone and toluene extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Concentrations are expressed per g lipid.*

Extract	Hexane/acetone		Toluene	
Contaminant group	Atlantic	Baltic	Atlantic	Baltic
2378TCDD, pg/g	11	95	6	62
Dioxins [a], pg/g	216	931	120	702
Furans [b], pg/g	3381	8397	1366	3275
PCBs [c], ng/g	819	7055	670	6798
OC pesticides [d], ng/g	1077	7211	747	7778
Coplanar PCBs, ng/g	10.5	79.4	6.7	66.5
Chlorinated napthalenes, ng/g	2.1	17.7	1.5	16.6
Total PCDE [e], pg/g	5349	17313	2460	9697
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Bioassays

Mortality was not increased by treatment in the first experiment, which is probably due to high background mortality. In the second experiment, dose-dependent mortality increases were observed with fraction IV of the Baltic extract and with fraction V of the Atlantic extract ($p<0.05$, table 2).

Table 2. *Mortality, number of surviving embryos, and frequency of malformed embryos after exposure to different fractions of extracts of guillemots from the Atlantic Ocean and the Baltic Sea. P-values indicate significant dose responses in mortality.*

Fraction	Dose beq	Mortality Atlantic	Baltic	Survivors, n Atlantic	Baltic	Malformations[a], % Atlantic	Baltic
Control	0	57.7		11		3	
I	0.03	10.0	37.5	9	5	10	0
	0.3	72.3	66.7	3	3	9.1	22.2
	3	50.0	75.0	5	2	30	25
II	0.03	30.3	33.3	7	6	0	22.2
	0.3	30.0	33.3	7	6	10	0
	3	25.0	41.7	6	7	12.5	16.7
III	0.03	71.4	50.0	2	3	0	0
	0.3	45.5	37.5	6	5	0	0
	3	33.3	20.0	6	8	0	20
Control	0	30.4		16		3	
IV	0.03	22.2	33.3	7	4	0	0
	0.3	14.3	37.5	6	5	0	0
	3	55.6	90.0	4	1	0	21.4
			p=0.002				
V	0.03	12.5	14.3	7	6	0	20
	0.3	57.1	0.0	3	10	14.3	0
	3	85.7	37.5	1	5	28.6	0
		p=0.009					
VI	0.03	33.3	20.0	4	8	0	10
	0.3	30.0	40.0	7	6	20	0
	3	10.0	22.2	9	8	0	0

[a], *numbers reflect total amounts, observed in dead and living embryos*

Malformations and morphometry

Embryos with malformations were observed after exposure to fractions I, II and V of both extracts, and in embryos exposed to fractions III and IV of the Baltic extract and fraction VI of the Atlantic extract (table 2). Malformations included edema, hemorrhaging, open body cavities, missing eyes, deformed bills, aberrant limbs and hydropericard, both in living and dead embryos. In order to perform regression analyses on the data with contaminant concentrations, exposure doses of contaminants were estimated from the extract analyses, in combination with the data from the salmon fraction analyses. In short, dividing the total amount of contaminants in the extract (the product of concentration and lipid content) by the number of birds from which the extract was derived yields the amount of contaminants in one adult bird body. A subsequent division by 10, as we assumed that the body burden of 1 bird equals the body birden of 10 eggs, yields the number of bird egg equivalents. Finally, contaminant doses of the different fractions were calculated by multiplication with the appropriate percentage, at which the contaminants were distributed over the fractions as measured for salmon extracts. From this, the amount of contaminants injected using 0.03, 0.3 or 3 beqs can be derived. Regression analysis under a binomial model, with log transformed contaminant concentrations, revealed PCBs ($p=0.022$) and PCDEs ($p=0.043$) as explanatory variables for total malformations (in both dead and living embryos). Data for fractions III and VI were included in the analysis.

No differences were found for embryo weight, yolk sac weight, or organ weights ($p>0.05$, tables 3 and 4). Therefore, differences between treatments for other parameters cannot be explained by differences in biometry.

Table 3. *Embryo weight of surviving embryos exposed to different fractions of extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Values are mean ± standard deviation, based on the number of survivors listed in table 2.*

Fraction	Dose	*Extract*	
	beq	Atlantic	Baltic
Control	0	24.3±2.5	
I	0.03	22.8±4.6	20.5±3.2
	0.3	24.2±4.3	21.1±5.1
	3	24.7±3.4	16.9±4.9
II	0.03	23.7±3.3	25.1±4.7
	0.3	22.1±4.7	22.4±2.9
	3	23.5±3.5	24.0±3.2
III	0.03	20.4±3.8	21.0±3.6
	0.3	25.4±1.8	23.8±3.3
	3	24.9±3.0	23.2±2.2
Control	0	24.4±3.5	
IV	0.03	24.7±4.2	24.7±2.2
	0.3	25.3±5.9	24.3±3.5
	3	27.2±2.1	27.2
V	0.03	25.9±1.8	26.2±2.5
	0.3	21.2±3.3	24.7±3.2
	3	21.3	22.9±1.4
VI	0.03	21.2±4.0	21.3±2.7
	0.3	23.1±3.0	26.4±2.1
	3	23.2±4.1	21.7±3.6

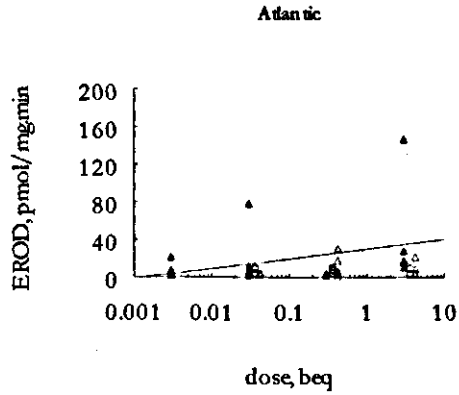
Table 4. *Organ weights of surviving embryos exposed to different fractions of guillemots from the Atlantic Ocean and the Baltic Sea. Values are mean ± standard deviation, based on the number of surviving embryos listed in table 2.*

Fraction	Dose	Liver		Heart		Brain	
	beq	Atlantic	Baltic	Atlantic	Baltic	Atlantic	Baltic
Control	0	548±91.4		237±31.4		792±66.6	
I	0.03	466±93.8	439±103.1	205±21.4	191±46.7	726±179.1	726±68.9
	0.3	507±117.0	430±130.8	188±28.7	203±32.1	785±96.4	760±75.5
	3	511±89.4	460±198.0	220±44.4	190±42.4	780±81.7	615±106.1
II	0.03	509±44.4	553±103.0	193±21.9	228±37.4	765±72.3	781±77.7
	0.3	437±130.1	487±50.4	201±38.3	214±58.7	755±70.4	757±71.5
	3	507±96.3	526±71.4	211±24.2	202±23.2	749±80.6	787±62.3
III	0.03	410±84.9	507±104.1	235±77.8	183±40.4	730±0	693±122.2
	0.3	546±46.3	534±86.5	210±18.3	224±32.9	826±46.5	800±51.5
	3	513±53.5	528±95.3	222±46.3	208±20.7	773±33.7	729±37.1
Control	0	541±81.6		219±29.1		761±58.9	
IV	0.03	534±58.8	550±62.3	201±21.3	229±10.4	787±96.0	754±56.0
	0.3	536±94.2	553±117.9	203±25.2	216±31.9	783±77.5	718±74.7
	3	574±50.8	548	233±28.1	223	794±58.0	727
V	0.03	570±51.9	534±96.5	199±15.0	212±19.2	806±71	806±27.4
	0.3	455±86.0	540±88.4	186±13.2	196±15.6	753±42.9	776±64.0
	3	508	503±52.1	225	228±14.6	713	745±48.1
VI	0.03	455±103.1	498±150.8	195±41.9	225±33.6	721±63.5	718±103.8
	0.3	515±93.8	580±61.2	187±37.1	228±29.6	774±67.5	793±77.9
	3	532±95.8	492±142.8	219±33.3	201±30.7	765±77.4	702±78.2

Enzyme activities

EROD activity was dose-dependently induced up to 7 times in fraction I of the Atlantic and up to 15 times in fraction I of the Baltic extract ($p<0.05$, figure 2). For the Atlantic extract, the dose dependent increase in EROD activity could be described by a linear relationship ($p=0.038$), but it should be noted that the observation at the highest dose (3 beq) was mainly responsible for this observation. The Baltic extract, however, resulted in a dose dependent induction of EROD activity that could be described by a logistic curve ($p=0.038$), with an induction of 13 times at 0.3 beq and an induction of 15 times at 3 beq. Fraction IV induced EROD activity up to 11 fold at 3 beq of the Atlantic extract, and up to 9 (0.3 beq) and 17 (3 beq) fold for the Baltic extract (figure 3). This was dose dependent and could be described by logistic curves ($p<0.001$). For the Atlantic extract, this was again mainly due to the observation at the highest dose. In fraction V of the Atlantic extract, an apparent induction of eight times was found at the dose of 3 beq ($p<0.001$, figure 3). However, this observation was only based on one surviving embryo.

Multiple regression analysis on the data with log transformed contaminant concentrations (calculated as described in the morphology paragraph) revealed that dioxins were the predominant descriptive variable for EROD activity ($p<0.001$). These compounds were expected to be practically completely allocated to fractions I and IV. The increase in EROD activity with contaminant exposure could be described by an exponential curve (EROD=5.89+23.09*3.775^log(dose), $p<0.001$).



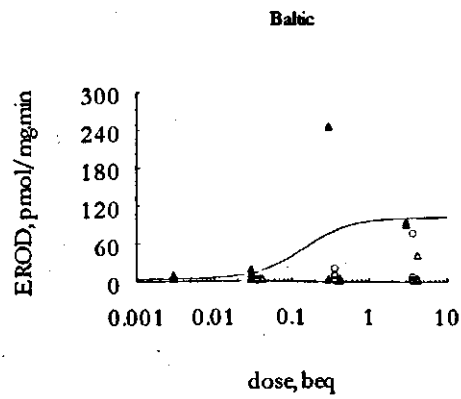


Figure 2. *EROD activity in different fractions of hexane/acetone extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Control values are shown at 0.003 beq on the x-axis. Filled triangles, fraction I; open cirkels, fraction II; open triangles, fraction III. Regression lines shown are significant (fraction I, Baltic logistic, p=0.038 and Atlantic linear at p=0.038).*

Table 5. *Thiamine dependent enzyme activities in embryos exposed to different fractions of extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Activities are expressed as % of controls, shown as mean ± standard deviation, based on the number of surviving embryos as listed in table 2. Transketolase and glucose-6-phosphate dehydrogenase (G6PDH) were measured in brain, and α-ketoglutarate dehydrogenase (α-KGDH) was measured in heart.*

Fraction	Dose	Transketolase		G6PDH		α-KGDH	
	beq	Atlantic	Baltic	Atlantic	Baltic	Atlantic	Baltic
I	0.03	85±61	117±52	100±34	127±68	117±100	113±92
	0.3	82±43	55±6	139±95	54±3	57±20	82±22
	3	106±73	158±123	106±56	158±117	58±38	30±43
II	0.03	79±36	81±38	95±49	89±34	104±41	113±100
	0.3	143±64	87±74	151±67	82±51	91±33	76±11
	3	83±53	105±72	82±32	134±92	87±37	111±56
III	0.03	52±15	74±23	53±37	71±35	91±11	58±33
	0.3	82±55	66±27	108±65	76±38	68±23	70±41
	3	86±35	121±60	91±59	83±49	68±20	65±43
IV	0.03	99±20	144±17	103±33	114±29	91±80	103±56
	0.3	148±47	109±30	98±16	108±36	81±19	114±19
	3	150±45	146	127±31	124	90±36	0
V	0.03	91±34	125±71	103±45	108±35	97±52	121±50
	0.3	151±110	167±73	127±65	121±33	171±72	121±64
	3	133	121±32	130	92±17	169	104±52
VI	0.03	96±26	91±22	82±15	103±15	45±26	90±38
	0.3	104±29	95±14	76±12	99±8	83±39	64±28
	3	113±28	106±24	81±23	92±17	82±67	107±34

Table 6. *Acetylcholinesterase activity in brains of embryos exposed to different fractions of extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Activities are expressed as % of controls, shown as mean ± standard deviation, based on the number of survivors listed in table 2.*

Fraction	Dose	*Extract*	
	Beq	Atlantic	Baltic
I	0.03	96±56	141±77
	0.3	107±60	63±20
	3	125±51	120±63
II	0.03	86±50	91±39
	0.3	169±66	100±72
	3	92±51	119±72
III	0.03	57±7	63±16
	0.3	85±36	72±15
	3	88±36	111±55
IV	0.03	90±13	103±33
	0.3	104±14	99±36
	3	108±30	108
V	0.03	89±13	107±26
	0.3	132±78	127±35
	3	126	93±13
VI	0.03	76±9	104±13
	0.3	88±14	120±10
	3	89±14	102±11

Table 7. *Hepatic porhyrins in embryos exposed to different fractions of extracts of guillemots from the Atlantic Ocean and the Baltic Sea. Levels are expressed as % of controls, shown as mean ± standard deviation, based on the number of surviving embryos as listed in table 2.*

Fraction	Dose	*Extract*	
	Beq	Atlantic	Baltic
I	0.03	92±52	73±17
	0.3	72±9	69±5
	3	64±27	118±96
II	0.03	66±18	57±33
	0.3	107±52	84±21
	3	84±16	72±10
III	0.03	90±4	50±8
	0.3	62±34	57±12
	3	62±16	69±13
IV	0.03	119±63	106±32
	0.3	98±59	119±65
	3	164±94	42
V	0.03	167±122	124±61
	0.3	71±68	99±64
	3	67	97±31
VI	0.03	90±47	104±22
	0.3	74±58	104±4
	3	86±35	106±21

Table 8. *Bursa index of embryos exposed to different fractions of guillemot extracts from the Atlantic Ocean and the Baltic Sea. Values shown are mean ± standard deviation. P-values indicate significant dose responses in bursa index.*

Fraction	Dose	*Extract* Atlantic		Baltic	
	beq	n	Index	n	Index
Control	0	13	1±0		
I	0.03	7	1±0	5	3.2±0.4
	0.3	3	1±0	2	3.5±0.7
	3	5	2.2±1.6	1	3±0
			p=0.011		*p<0.001*
II	0.03	6	1±0	5	1.4±0.9
	0.3	7	1.7±1.3	5	2.8±1.3
	3	5	1±0	6	2.8±0.4
					p<0.001
III	0.03	1	1	1	2
	0.3	4	1±0	2	1±0
	3	6	1±0	6	1±0
Control	0	9	1.3±1		
IV	0.03	7	2.1±1.1	4	1.5±1
	0.3	6	1.7±1.5	5	2.6±1.5
	3	4	1.5±1	0	*p=0.070*
V	0.03	7	1.3±0.8	6	1.3±0.8
	0.3	3	2±1.7	9	1.7±1.3
	3	1	4±0	5	1±0
			p=0.080		
VI	0.03	4	1.5±1	8	1.3±0.5
	0.3	7	1±0	4	1±0
	3	8	1.5±0.9	8	1.1±0.2

Discussion

The aim of the present study was to assess the current pressure of contaminants on the common guillemot living in the Baltic Sea. The results of the chicken embryo bioassay, in which fractions of whole body extracts were tested, were used to determine which class of contaminants may evoke toxic effects in the common guillemot. Within the framework of interpreting the data, a comparison between field studies on wild birds and contaminant concentrations in the guillemot extracts (recalculated into egg equivalents) from the present study was made and will be described here.

The most important observation in this study was that fractions I and IV induced EROD activity in livers of exposed chicken embryos and altered bursa morphology, at a dose of 0.3 beq of the Baltic extract. EROD induction was significantly induced by the corresponding fractions of the Atlantic extract, but only at the highest dose applied (3 beq). Thus, in the Baltic extract effects were observed at levels which reflected only 3% of the adult body burden, indicating that guillemots in the Baltic Sea may be confronted with an ongoing threat by contaminants. Histological examination of the bursa of Fabricius confirmed this observation, as decreased lymphocyte density was found in organs of embryos exposed to fractions I, II, IV and V, which may have immunotoxic consequences (Hoffman *et al.*, 1996; Grasman and Whitacre, 2001). In consistency with the results on EROD activity, a significant effect on lymphocyte density was observed at a dose of 0.3 beq of Baltic fractions I and IV. Fractions I and IV were expected to contain the majority of the contaminants with capacity to bind to the Ah receptor, present in the total extract. In fact, total amount of dioxin like contaminants correlated well with the induction of EROD activity ($p<0.001$). The induction seen in embryos exposed to fraction V of the Atlantic extract is rather surprising, as no induction was observed in the corresponding fraction of the Baltic extract, but it should be noted that this observation was only based on 1 individual and it cannot be excluded that this particular one is an outlier. Biological variation has been observed to be rather high (as can be seen from figures 2 and 3).
Effects on the bursa may have been caused by dioxins and coplanar PCBs, present in fractions I, II, IV and V ($p=0.001$). Toxic potency of these fractions was confirmed by high mortality rates among exposed embryos, as well as by a significantly increasing occurrence of malformations. The malformations in embryos exposed to fractions III (Baltic extract) and VI (Atlantic extract) could not be explained by PCBs, PCDEs or dioxins, as only traces of these compounds were present in these fractions. However, these data were included in the statistical analysis; the fact that PCBs and PCDEs were found to be the main explaining factors indicate that the occurrence of malformations in fractions III and VI may have been a random event, as in the controls. The observation of effects in embryos exposed to corresponding fractions of the different extracts (i.e., I and IV, II and V) was not surprising, as the contaminant compositions of the whole extracts were very similar.

No other effects were observed in the present study. Thiamine dependent enzymes were not decreased in their activity, which is in line with an earlier study in which several model compounds were tested for their effect on transketolase (chapter 4). The increased activity of glucose-6-phosphate, as expected from the hypothesised mechanism in which induced EROD activity is linked via this enzyme to thiamine deficiency, was not found in the present experiment. Therefore, it seems to be highly improbable that induced thiamine deficiency plays a role in embryotoxicity in birds.

Inhibition of the neurotransmitter acetylcholinesterase does not seem to play a role either, as none of the fractions changed the activity of this enzyme. In addition, the heme biosynthetic pathway was found to be intact, as no accumulation of hepatic porphyrins was observed in these chicken embryos. Although porphyrin accumulation has been described for contaminants that also induce EROD activity, it has been shown to be a less sensitive parameter in the chicken embryo (Rifkind *et al.*, 1985), which is in line with our present observations that hepatic porphyrins did not accumulate. Although high mortality rates implicate that numbers of surviving embryos were sometimes very low and statistical power is reduced, it is not likely that differences between groups for any of the tested parameters were left undetected. All variables measured showed high variability both within and between groups, and were always within the same range as the controls, as determined in previous experiments, except for EROD activity.

As the bioassay used in the present study applies a species that is known to be more sensitive than fish-eating birds (Bosveld *et al.*, 1995; Sanderson and Bellward, 1995; Kennedy *et al.*, 1996), conclusions on the present contamination pressure on the common guillemot cannot be drawn without a comparison to field studies. Concentrations in the guillemot extracts and comparison to concentrations in the field situation may add to a better risk assessment. The concentrations of dioxins and PCBs in the extracts were calculated to beqs as described in the malformations section above. For the Baltic hexane/acetone and toluene extract, 1 beq dioxins equals approximately 2.6 and 1.9 ng/egg, respectively. For the Atlantic hexane/acetone and toluene extract, 1 beq dioxins is approximately 0.8 and 0.5 ng/egg, respectively. PCBs are estimated to be present at levels of 19 µg/egg in both extracts of the Baltic guillemots and at levels of 3 µg/egg in extracts of Atlantic guillemots. Levels in eggs can be converted to concentrations in bird embryos by the assumption that 20% of the amount of contaminants present in the yolk is absorbed by the 19 day old embryo (as described for PCBs in chapter 3) and using an average embryo weight of 15 gram (derived from the equation in Masters Vleck and Vleck, 1987). Then, dioxin concentrations in guillemot embryos exposed to Baltic extracts would be estimated to be approximately 0.03 ng/g and in embryos exposed to Atlantic extracts this would approximate 0.01 ng/g. PCB concentrations would be 230 and 30-40 ng/g in embryos exposed to Baltic or Atlantic extract, respectively. These estimations allow for comparison to field studies on fish-eating birds. In general, it is noted that levels measured in the extracts were lower than those associated with biomarker responses in field situations reported for other species. One study reports on great blue heron chicks with TCDD concentrations only three times the levels expected in the present study, which showed a 2.5 fold induction of EROD activity (Bellward *et al.*, 1990). Other studies indicate that EROD induction in wild birds is generally observed at much higher levels of contaminants: several authors report induction of EROD activity in great blue herons, black crowned night herons, Forster's tern or bald eagles, at contaminant levels more than 15 to over 300 times those estimated in the present study (e.g. Hoffman *et al.*, 1987; Sanderson *et al.*, 1994; Rattner *et al.*, 1993, 1994 and 1997; Eliott *et al.*, 1996; Custer *et al.*, 2001). There is only limited information on the relation between induced EROD activity and possible toxic end points; from the mentioned studies, only two found a small increase in abnormalities (Hoffman *et al.*, 1987; Sanderson *et al.*, 1994). Custer *et al.* (1999) studied reproductive succes of double-crested cormorants from Green Bay, and found a strong correlation between PCBs and EROD activity; however, none of the measured

parameters of reproductive output correlated with EROD. Thus, there is no direct evidence for a causative relationship between EROD induction and toxic effects in these birds. There are some studies in which contaminant concentrations are related to toxic end points. Larson *et al.* (1996) found a significant, PCB related difference in hatching success and occurrence of chicks with misformed bills in a population of double-crested cormorants in Lake Michigan. Wet weight concentrations of PCBs in that study were about 20 times the estimated egg concentrations, that were derived from lipid based concentrations (which are generally higher than concentrations based on wet weight) in the guillemot extracts in the present study. The concentrations of PCBs in the present study are comparable to the lowest concentrations measured in double-crested cormorant eggs in the Great Lakes; in that study, 10 fold higher concentrations correlated with an increased mortality rate among embryos (Tillitt *et al.*, 1992). In addition, total PCBs were found to be correlated with hatching and deformity rates in double-crested cormorants and caspian terns from the Great Lakes (Ludwig *et al.*, 1996), at levels only slightly higher than those measured by Tillit *et al.* (1992). Thus, concentrations of contaminants seem to be too low to induce EROD activity, but may be close to concentrations related to possible toxic effects in wild birds. As long as the relative sensitivity of the Baltic guillemot remains unknown, however, there is no strong evidence that such effects will occur.

The bioassay used in the present study indicated the possible immunotoxic potency of contaminants present in fractions I, II, IV and V. Effects on the bursa of Fabricius have been described in wild birds exposed to several contaminants. For instance, a decreased weight of the bursa was reported for double-crested cormorants exposed to TCDD and PCB 126, and for common tern chicks exposed to different contaminants via the food (Powell *et al.*, 1998; Bosveld *et al.*, 2000). However, levels of contaminants were much higher than the ones estimated from the present study. Decreases in lymphocyte density in the bursa appear to be more sensitive than organ weight, although this has only been studied in chicken embryos (Nikolaidis *et al.*, 1988a and 1988b; Andersson *et al.*, 1991; Hoffman *et al.*, 1996; Grasman and Whitacre, 2001). In fact, contaminant levels associated with this response in chicken are comparable to those applied in the present study. However, as the relative sensitivity of guillemots is not known at this moment, no conclusions can be drawn on the actual threat that contaminants pose on their immunocompetence.

Finally, a comparison on effective contaminant concentrations is made here for porphyrin accumulation. Although porphyrin accumulation was not observed in the chicken embryo assay, a risk estimate should be made, as there is not much information on species differences in sensitivity. The chicken embryo has been shown not to be unequivocally the most sensitive species (Sanderson *et al.*, 1998). Porphyrin accumulation has been described in birds experimentally exposed to PCBs and hexachlorobenzene at concentrations as high as 500 mg/kg food (Carpenter *et al.*, 1985a; Miranda *et al.*, 1987), resulting in a residue level of 50 µg PCBs/g liver (Elliott *et al.*, 1990). A field study on herring gulls revealed significant differences in hepatic porphyrins between contaminated sites and the reference site, at hepatic concentrations of 35 µg/g PCBs and 0.8 ng/g dioxins (Fox *et al.*, 1998). It is clear from these data that concentration estimates from the present study are lower than the concentrations associated with disturbance of the heme biosynthetic pathway. This assumption is strengthened by the study of Bellward *et al.* (1990), in which EROD induction was observed at levels twice those expected in the present study, but without effects on hepatic porphyrins. Although to date, no information is available on the porphyrinogenic sensitivity of the common guillemot, species do not seem to

show large variability, as demonstrated by the *in vitro* study by Sanderson *et al.* (1998). Therefore, it is not likely that guillemots will face disturbances of their heme biosynthetic pathway.

In summary, guillemot extracts tested in the chicken embryo assay induced EROD activity up to 17 fold, enhanced the occurrence of abnormal embryos and changed the morphology of the bursa of Fabricius. None of the other six end points were affected; no effects were observed on morphometry, thiamine dependent enzymes, glucose-6-phosphate dehydrogenase, acetylcholinesterase or porphyrin levels. The bioassay was used in order to find the contaminant classes that resulted in toxicity. The fractions that were observed to induce biomarker responses and cause toxic effects (mortality and malformations) in the bioassay were fractions I, II, IV and V; these are the fractions that are expected to contain the majority of the known lipophilic POPs (based on analyses of similar fractions of salmon extracts).
Concentrations found in the extracts of guillemots from both the Atlantic Ocean and the Baltic Sea were found to be lower than concentrations associated with biomarker effects in wild birds as reported before. Effects on immunocompetence in the guillemot could not be excluded.

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CHAPTER 6

EFFECTS OF AN EXTRACT OF OYSTERCATCHER EGGS FROM THE ZEEHAVENKANAAL IN THE NETHERLANDS, AND OF ITS MAJOR CONTAMINANT, HEXACHLOROBENZENE, ON THE CHICKEN EMBRYO

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Abstract
The sediment of the canal 'Zeehavenkanaal' in the Netherlands contains high concentrations of contaminants, especially hexachlorobenzene, and it has been shown that oystercatchers foraging on the canal accumulate appreciable amounts of the contaminants from the sediment. The present study was performed to assess the embryotoxic effects of these contaminants. To this end, a two step approach was followed. In step one, the toxic effects of hexachlorobenzene were studied after injection of the single compound into fertilised chicken eggs prior to incubation at concentrations realistic for the field situation. In step two, yolks of oystercatcher eggs were extracted and the embryotoxic potency of this extract was studied in the same bioassay, using doses of 1%, 10% and 100% of the contaminant load in one average egg. The extract contained hexachlorobenzene, PCBs and possibly other, unknown compounds. Hexachlorobenzene did not affect survival or biometry, but induced a nonsignificant decrease in lymphocyte density in the bursa of Fabricius. The egg extract caused an induction of EROD activity at the highest dose applied, and decreased lymphocyte density in the bursa of Fabricius. These results indicate that oystercatcher embryos from the Zeehavenkanaal may be at risk for effects of the contaminants present in the sediment of the canal, which accumulate in the eggs.

Introduction
In the past, measurements on sediment from the Zeehavenkanaal in the Netherlands revealed high concentrations of contaminants, especially hexachlorobenzene. Oystercatchers that forage in this area have been found to accumulate these contaminants, which are deposited in the eggs (Eggens *et al.*, 2000). Although there is no evidence that the population is compromised by toxic effects, the high levels of contamination were considered reason for concern. Hexachlorobenzene has been shown to decrease survival and hatchability of quail embryos (Schwetz *et al.*, 1974), and has been associated with decreased reproductive success in wild birds (Vos *et al.*, 1969; Gilbertson and Reynolds, 1972; Jarman *et al.*, 1996). The compound is known to induce porphyrin accumulation in birds, which is an indication of disturbed heme biosynthesis (Vos *et al.*, 1971; Carpenter *et al.*, 1985a). Eventually, a disturbance of the heme biosynthesis may lead to disfunctioning of hemoproteins such as hemoglobulin and cytochromes (Marks, 1985). In addition, hexachlorobenzene has been described to affect the activity of the cytochrome P450 system, as well as to affect parameters that are related to immunocompetence (Carpenter *et al.*, 1985a, 1985b; Schielen *et al.*, 1995; Machala *et al.*, 1996). The aim of the present study was to assess the embryotoxic effects of contaminants present in the oystercatcher eggs from the Zeehavenkanaal. To this end, a two step approach was followed. The chicken embryo bioassay was used to assess the toxic effects of hexachlorobenzene and of egg extracts on the chicken embryo. The first step involved the injection of the single compound, hexachlorobenzene, into the yolk of fertilised chicken eggs prior to incubation, at concentrations that were based on measured concentrations in the field situation from the previous year. The second step consisted of the assessment of possible toxic effects of the complex mixture of contaminants present in the oystercatcher eggs. To this end, yolks from eggs collected in the colony of oystercatchers foraging in the Zeehavenkanaal area were extracted with hexane and the extracts were tested for embryotoxic effects in the chicken embryo bioassay. In this experiment, injected portions of the extract reflected 1, 10 or 100% of the amount of contaminants present in the average egg. Embryos were examined for

abnormalities, and EROD activity and porphyrin concentration were measured in the livers. In addition, the bursa of Fabricius was examined histologically. This organ was chosen for studying the effects of contaminants on the immune system, as it has been described that B lymphocytes are more sensitive towards the toxic action of contaminants than T lymphocytes (Harris *et al.*, 1976; Nikolaidis *et al.*, 1988a, 1988b and 1990; Brunström *et al.*, 1992; Wilmer *et al.*, 1992; Hoffman *et al.*, 1996). The extract was analysed for hexachlorobenzene; in addition, PCBs were analysed, since they are often found concurrent with hexachlorobenzene, and have been associated with toxic effects in birds (*e.g.* Gilbertson *et al.*, 1991).

Materials and Methods

Extract preparation and chemical analysis

Eggs collected in 1999 were analysed for hexachlorobenzene. Fresh eggs were homogenized, blended with an internal standard and dried by stirring with sodium sulphate. The mixture was cleaned over a silica column, which was composed of two deactivated silica gel phases (10% water and 40% sulphuric acid), using n-hexane-dichloromethane (8:2) as an eluent. The solvent was evaporated to dryness and the residue was redissolved in toluene. The samples were analysed by GC/MSD, with single ion monitoring.

Extracts for study in the bioassay were obtained from oystercatcher eggs collected in May 2001, which were kept frozen at -20°C until extraction. The yolks of 15 eggs were pooled and dehydrated with sodiumsulphate. All solvents used were of multigrade quality. The mixture was then soxhlet extracted in hexane. The solvent was evaporated to dryness using rotation evaporation. The remaining lipids were extracted twice by liquid extraction with acetonitrile. The two acetonitrile fractions were combined and the solvent was removed using rotation evaporation. The extract was then redissolved in hexane and cleaned up over an aluminum oxide column as described by van den Brink (1997), using hexane as eluens. The final extract was analysed for hexachlorobenzene and PCBs using GC/MSD, in selected ion mode. Quantification was based on external standard samples, using hexachlorobenzene and PCBs 28, 31, 49, 52, 66, 95, 101, 105, 118, 138, 141, 149, 151, 153, 170, 180, 183, 187, 194, 206, 209.

Bioassays

Fertilised chicken eggs were kindly provided by Verbeek's Poultry farm, Lunteren.

Hexachlorobenzene (purity 99.8±0.1%) was obtained from Promochem (the Netherlands). Injection solutions of hexachlorobenzene were prepared in sterilised propylene glycol; concentrations were based on data from the egg analyses described above, which averaged 78 µg/kg, with a range of 29-147 µg/kg (table 1). Thus, the injection concentrations were 0.0123 to 0.184 mg/ml, resulting in doses of 0.615, 2.075, 4.25, 6.7 and 9.2 µg/egg (which is similar to 11, 38, 77, 122 and 167 ppm, respectively). Each concentration was injected in ten eggs; in addition, ten eggs were injected with the carrier (controls).

Injection solutions of the extract were prepared in sterilized propylene glycol. A proportion of 1, 10 or 100% of the contaminants present in one average egg was dissolved per 50 µl (the volume for one injection). Before addition of propylene glycol, hexane was removed by evaporation under sterile conditions. Thus, eggs were injected with 0.01, 0.1 or 1 bird egg equivalent (beq). Since the fresh weight of a

chicken egg is comparable to that of an oystercatcher egg, the concentration of 1 beq was comparable to the average egg concentration in the field situation.
The injections were performed according to the method described in chapter 2. After injection, eggs were incubated for 19 days at 37.5±0.5°C and 50-60% relative humidity. Survival was recorded and dead and non fertile eggs were discarded. At day 19, embryos were examined for gross abnormalities, weighed and decapitated. Livers were dissected, frozen in liquid nitrogen and stored at -80°C until further processing. Bursae of Fabricius were dissected and fixed in unifix.

Enzyme assays
Ethoxyresorufin-*O*-deethylase (EROD) activity was measured in liver microsomes as described by Kennedy *et al.* (1993). In short, livers were homogenised in phosphate buffer (0.125 M, pH 7.6) containing EDTA (0.1 M). Subsamples of the homogenates were stored at -80°C for porphyrin analysis, and the rest was centrifuged at 15,000 *g* for 12 minutes at 4°C. The supernatants were centrifuged at 150,000 *g* for 72 minutes at 4°C, and the pellets were resuspended in phosphate buffer with 20% glycerol. The microsomes were stored at -80°C until analysis. For the EROD assay, 10 µl of microsomes were added to 220 µl buffer containing tris-HCl (80 mM, pH 8), NaCl (80 mM), bovine serum albumine (1 mg/ml) and 7-ethoxyresorufin (1.1 µM). The reaction was started by the addition of 20 µl NADPH (3mM), and the production of resorufin was measured fluorimetrically over 15 minutes at wavelengths of 530/590nm excitation/emission, at 37°C. Protein concentration in the microsomes was measured on multiwell plates using the BCA protein assay reagent of Pierce. After the addition of 200 µl of the reagent to diluted samples, the plate was incubated at 37°C for 30 minutes. Then, the plate was cooled down to room temperature and absorbance was measured against a BSA standard in a spectrophotometer (Versamax, Applied Biosystems) at a wavelength of 562 nm.

Hepatic porphyrins
Porphyrins were measured fluorimetrically in multiwell plates. Liver homogenates (from the liver microsomal preparation) were diluted in water and extracted using a buffer containing tris-HCl (18.75 mM, pH 8.0) and trichloroacetic acid (15.6%). Then, 180 µl of the extracts were added to 20 µl NaCl (0.9%), the plate was shaken and placed on a UV lamp for 5 minutes, after which it was centrifuged at 5,000 *g*. The supernatants were transferred to a new plate, which was measured at 400/590nm excitation/emission. Porphyrin levels were calculated in µg per g liver.

Histology
The bursae of Fabricius were dehydrated through an increasing ethanol gradient and embedded in paraffin. Semithin (4-6 µm) slices were cut on a microtome and stained using haematoxiline/eosine. Slices were studied double blind under the microscope and appearance of the bursa of Fabricius was classified according to observed lymphocyte density. The appearance of follicles was graded along the axis of normal, with high lymphocyte density (class 1) to clearly abnormal, with decreased lymphocyte density and lighter staining (class 4), with two intermediate classes. The average index was then calculated per group.

Statistics
Statistical analyses were performed under Genstat 5.5. Mortality rates, occurrence of malformations and histological index were tested with a general regression model

after logit transformation of the data and under a binomial distribution. Histological index was tested with a one-sided significance level of 10%, as the expected effect was an increase in bursal index after exposure to the contaminants: in a previous study (chapter 5), the control situation was designated as class 1, and therefore, a response could, per definition, only be measured from an increase in bursa index. For the other tests, α was stated at 5%. ANOVA was used to detect differences between groups for organ weights, enzyme activities or porphyrin levels as compared to control values. Regression analysis of maximum likelyhood was performed on the data with log transformed doses to detect dose-response relationships.

Results

Chemical analysis of oystercatcher eggs

In 1999, oystercatcher eggs contained 29-147 µg/kg hexachlorobenzene (table 1). In 2001, chemical analysis revealed that 5 ml extract contained 6.4 µg/ml HCB. As the extract resulted from 15 egg yolks, this means that one egg (1.0 beq) contained on average 2 µg HCB (table 2). Total PCBs were present at a concentration of 25 µg/egg (table 2).

Table 1. *Hexachlorobenzene concentrations in oystercatcher eggs collected from the Zeehavenkanaal area in 1999, and concentrations of hexachlorobenzene used in the injection experiment with the single compound.*

Measured concentration, 1999 ppm	Injected concentration, ppm	µg/egg
	0	0
29 (low)	11	0.615
	38	2.075
78 (average)	77	4.25
	122	6.7
147 (high)	167	9.2

Table 2. *Concentrations of contaminants measured in oystercatcher eggs collected from the Zeehavenkanaal area in 2001.*

Contaminant	Concentration µg/egg	Contaminant	Concentration µg/egg
HCB	2.13	PCB138	6.19
ΣPCBs	24.5	PCB141	0.04
		PCB149	0.30
PCB28	0.18	PCB151	0.06
PCB31	0.02	PCB153	8.81
PCB49	0.02	PCB170	0.88
PCB52	0.02	PCB180	2.22
PCB66	0.37	PCB183	0.47
PCB95	0.04	PCB187	2.25
PCB101	0.15	PCB194	0.15
PCB105	0.36	PCB206	0.03
PCB118	1.84	PCB209	0.08

Effects of hexachlorobenzene
Mortality was not increased after exposure to hexachlorobenzene (p=0.171; table 3a). Malformations were observed in embryos in the four lowest dose groups (table 3a). These included crossed bills, open body cavity, missing skull and missing eyes. No abnormalities were observed in the two highest dose groups.
No effects were found on embryo, yolk sac or organ weights (p>0.1; table 4a).
EROD activity was not changed after exposure to any dose of hexachlorobenzene (p=0.392; figure 1a). Accumulation of porphyrin was not observed in the livers of exposed embryos (p=0.081; figure 2a). The average lymphocyte density in the bursa of Fabricius showed a non significant decrease (p=0.53; table 5a).

Table 3a. *Number of fertile and viable eggs, mortality (%) and abnormalities (%) among 19 day old chicken embryos exposed to hexachlorobenzene.*

Dose, µg/egg	Mortality, %	$N_{living}/N_{fertile}$	Abnormalities, %
0	60	4/10	10
0.615	11	8/9	11.1
2.075	66	3/9	33.3
4.25	75	2/8	12.5
6.7	71	2/7	0
9.2	86	1/7	0

Table 3b. *Number of fertile and viable eggs, mortality (%) and abnormalities (%) among 19 day old chicken embryos exposed to extract of oystercatcher eggs.*

Dose, beq	Mortality, %	$N_{living}/N_{fertile}$	Abnormalities, %
0	10	9/10	0
0.01	25	6/8	0
0.1	50	4/8	50
1	18	9/11	27

Effects of oystercatcher egg extracts
Mortality among exposed embryos was not different from controls (p=0.325; table 3b), and varied between 10% and 50%. Malformations were found among embryos exposed to 0.1 and 1 beq of the extract (table 3b). These malformations included hemorrhages, overstretched toes, snake neck, missing skull and hydropericard. No differences were found for embryo, yolk sac or organ weights (p>0.1; table 4b).
EROD activity was significantly different in embryos exposed to 1 beq of the extract (p=0.008). The dose dependent increase could be described by an exponential curve (EROD=5.515*57306355^log(beq)+1.926; p=0.003; figure 1b).

Hepatic porphyrin concentrations did not differ between groups (p=0.141; figure 2b).
The structure of the bursa of Fabricius was different in exposed embryos as compared to controls. The normal appearance was mainly limited to the control group and decreases in lymphocyte density were observed in exposed embryos. Histological examination of the bursa of Fabricius revealed a dose dependent increase in bursa index (p=0.002; table 5b).

Table 4a. *Biometric parameters (average ± standard error) of 19 day old chicken embryos exposed to hexachlorobenzene. Values are based on the number of surviving embryos as listed in table 3a.*

Dose, µg/egg	Embryo, g	Yolk sac, g	Liver, mg	Bursa of Fabricius, mg
0	19.6±2.9	11.7±0.7	358±85	23.0±9.1
0.615	20.5±1.9	10.0±0.7	333±38	13.0±5.4
2.075	16.5±4.5	11.1±1.2	297±111	11.0±3.5
4.25	18.9±2.5	11.4±1.4	364±15	13.5±3.5
6.7	22.8±0.7	11.3±4.9	410±47	20.5±0.5
9.2	15.6	7.3	153	13.0

Table 4b. *Biometric parameters (average ± standard error) of 19 day old chicken embryos exposed to extract of oystercatcher eggs. Values are based on the number of surviving embryos as listed in table 3b.*

Dose, beq	Embryo, g	Yolk sac, g	Liver, mg	Bursa of Fabricius, mg
0	23.6±3.6	13.2±2.6	521±81	27.8±8.2
0.01	25.4±5.6	11.5±3.6	526±108	31.0±6.7
0.1	22.8±3.1	12.1±3.3	500±64	29.0±11.2
1	23.6±5.8	12.2±2.0	507±140	26.0±11.6

Table 5a. *Bursa index in surviving embryos exposed to increasing concentrations of hexachlorobenzene. Values are average ± standard deviation; 1=normal, and 4=abnormal; 2 and 3 are intermediates.*

Dose, µg/egg	Bursa index
0	2 ± 1
0.615	2.8 ± 0.8
2.075	3.5 ± 0.7
4.25	3 ± 0
6.7	2 ± 0
9.2	4 ± 0

Table 5b. *Bursa index in surviving embryos exposed to increasing doses of oystercatcher egg extract. Values are average ± standard deviation; 1=normal, and 4=abnormal; 2 and 3 are intermediates.*

Dose, µg/egg	Bursa index
0	1.2 ± 0.4
0.01	2.3 ± 0.8
0.1	1.8 ± 1.0
1	3.0 ± 1.0
	*p=0.002**

* *P-value indicates the significance level for the observed dose dependent increase.*

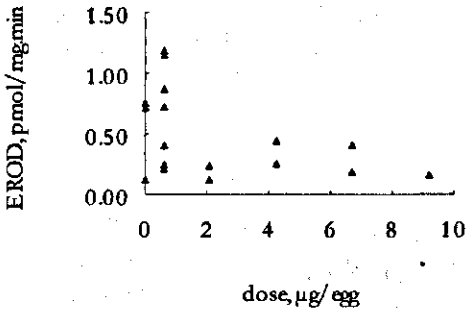

Figure 1a. EROD activity in livers of 19 day old embryos exposed to increasing concentrations of hexachlorobenzene.

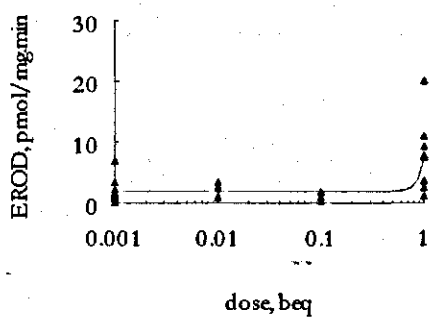

Figure 1b. EROD activity in livers of 19 day old embryos exposed to increasing doses of extract of oystercatcher eggs. Controls are shown at 0.001 beq on the x-axis. Curve fit: $EROD=5.515*5730635^{5^{log(beq)}}+1.926$; p=0.003.

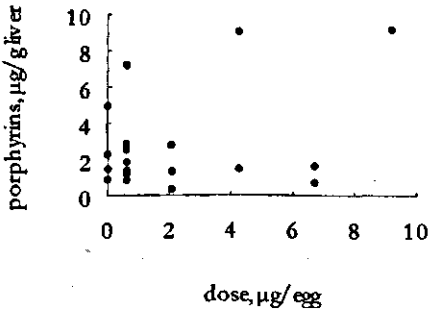

Figure 2a. *Hepatic porphyrin concentration in 19 day old embryos exposed to increasing concentrations of hexachlorobenzene.*

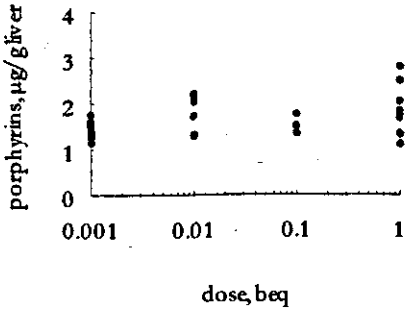

Figure 2b. *Hepatic porphyrin concentration in 19 day old embryos exposed to increasing doses of extract of oystercatcher eggs.Controls are shown at 0.001 beq on the x-axis.*

Discussion

In order to make a risk estimation for the oystercatcher living in the Zeehavenkanaal area, the results from the bioassay will be discussed in the framework of interspecies differences in sensitivity. In addition, a comparison will be made between the concentrations found in the present study and those reported for field studies in which toxic effects in wild birds have been found.

Mortality was high after exposure to hexachlorobenzene, but this was not observed after exposure to the oystercatcher egg extracts. This difference may in part be explained by interexperimental differences: background mortality was much higher in the hexachlorobenzene experiment. In addition, in the extract experiment, the highest dose injected was 1 beq, corresponding to 2.13 µg hexachlorobenzene/egg; this was comparable to the second lowest dose of 2.08 µg/egg in the hexachlorobenzene experiment, which was below the lethal concentration. In Japanese quail embryos, hexachlorobenzene caused increased mortality at concentrations of 6.2 µg/g in the eggs (Schwetz *et al.*, 1974; Vos *et al.*, 1969). The presence of PCBs in the extracts did not enhance the lethal toxicity of the mixture: in previous experiments, mortality was only increased after exposure to 60 µg PCBs/egg, approximately three times the concentration found in the present experiment (chapter 5). Thus, the concentrations of the contaminants present in an average oystercathcer egg were apparently below the toxic threshold.

The observation that embryo, yolk sac or organ weights were not affected by hexachlorobenzene or the extract shows that the contaminants did not have adverse effects on embryonic growth at the current concentrations. This is in line with another study, in which chicken embryos were exposed to extracts of heavily polluted Baltic guillemots; in that study, concentrations in chicken eggs were higher than in the present study (PCBs: ±7 µg/g lipids; dioxins: 1-8 ng/g lipids; hexachlorobenzene: 430 ng/g lipids), but no effects were found on biometric parameters (chapter 5).

The malformations observed in the two egg injection experiments were various, but all comply with the malformations described for complex mixtures of contaminants in wild birds (e.g. Gilbertson *et al.*, 1991) and chicken embryos (chapter 5). The difference between the two experiments may rely in the different contaminant compositions used. There was no dose dependent increase in the occurrence of malformations and the highest doses of hexachlorobenzene did not show any abnormalities at all. In a previous study, in which chicken embryos were exposed to extracts of Baltic guillemots containing higher concentrations of contaminants, there was a significant relation between the incidence of malformations and contaminant concentration (chapter 5). From the literature it is clear that the chicken embryo is by far the most sensitive species towards the toxic action of chlorinated hydrocarbons that result in increased incidence of malformations and decreased survival (Brunström and Reutergård, 1986; Brunström, 1988; Bosveld, 1995; Hoffman *et al.*, 1998), indicating that observed effects may not be extrapolated directly to wild bird species. However, since no effects on mortality were observed, it is not probable that oystercatcher embryos will suffer from enhanced mortality or malformations under the present contaminant levels. When focussing on PCBs, indeed, levels associated with increased mortality or malformations in caspian terns and double-crested cormorants in the field situation were much higher than those measured in the present study (Tillitt *et al.*, 1992; Ludwig *et al.*, 1996).

EROD activity was not induced after exposure to pure hexachlorobenzene. This was not surprising, as Machala *et al.* (1996) described the contaminant to be a weak inducer (the induction by hexachlorobenzene was only one third of that by TCDD, at a concentration that was 20.000x higher). At the highest dose level in the extract experiment, however, EROD activity was significantly induced. This can be explained by the PCBs present in the extract. Although the observed induction of EROD activity indicates the toxic potenty of the contaminants present in the oystercatcher eggs, the implication of this observation for the oystercatcher is not directly clear. In comparison to wild birds, the chicken embryo is known to be much more sensitive towards EROD induction; in common terns, the same level of induction was obtained at concentrations of PCB126 that were 250 times higher than that in chickens (Brunström and Halldin, 1998). In primary hepatocytes of different species, effective concentrations for a similar level of induction turned out to be different among wild bird species as well: the effective concentration of PCB126 was 80 times higher in common tern than in chicken, while it was even 450 times higher in the herring gull (Bosveld, 1995). The great blue heron and the cormorant were found to be 10-100 times less sensitive than the chicken (Sanderson and Bellward, 1995). The relative sensitivity of the oystercatcher as compared to the chicken remains to be determined. However, interspecies differences in sensitivity appear to be greatest between chicken and wild birds, while interspecies differences between wild birds are smaller. This suggests a sensitivity of oystercatchers to PCBs which is at least 10 times less than the chicken embryo. In combination with the observation that 0.1 beq did not induce EROD activity in the present study, this indicates that it is not likely that oystercatchers from the Zeehavenkanaal area will face enhanced EROD activity. This conclusion is supported by the fact that concentrations of contaminants associated with EROD induction in wild bird species in the field situation are generally higher than those measured in the present study. For instance, EROD induction was observed in great blue herons, black crowned night herons, Forster's terns and bald eagles, at contaminant levels 15 to over 300 times higher than the concentration in the oystercatcher eggs (e.g. Hoffman *et al.*, 1987; Sanderson *et al.*, 1994; Rattner *et al.*, 1993, 1994 and 1997; Elliott *et al.*, 1996; Custer *et al.*, 2001).

Porphyrin accumulation is a well known effect of hexachlorobenzene and PCBs (Vos and Koeman, 1970, Vos *et al.*, 1971; Carpenter *et al.*, 1985a; Miranda *et al.*, 1987). The concentrations of porphyrinogenic contaminants in oystercatcher eggs however, were apparently too low to induce these effects in the chicken embryo bioassay. Hepatic accumulation of porphyrins was found in birds experimentally exposed to PCBs and hexachlorobenzene at concentrations as high as 500 mg/kg food (Carpenter *et al.*, 1985a; Miranda *et al.*, 1987), which, for PCBs, resulted in a residue level of 50 µg /g liver (Elliott *et al.*, 1990). Interspecific differences in sensitivity to porphyrin accumulation are largely unknown, but the chicken embryo has been shown not to be unequivocally the most sensitive species as it has been for EROD induction (Sanderson *et al.*, 1998). Therefore, conclusions on expected effects in oystercatchers in the field situation cannot be drawn directly from the bioassay. However, a field study on herring gulls revealed significant differences in hepatic porphyrins between contaminated sites and the reference site, at hepatic concentrations of 35 µg/g PCBs and 0.8 ng/g dioxins (Fox *et al.*, 1998). From these data, it is clear that the current concentrations of contaminants in oystercatcher eggs from the Zeehavenkanaal area are below the concentrations associated with disturbance of the heme biosynthesis: the liver of a 19 day old embryo weighs approximately 400 mg, implying that at 35

µg PCBs/g liver, the total amount of PCBs present in the liver is 14 µg. This is higher than the calculated amount of PCBs taken up by the embryo on day 19, which consitutes 20% of the amount present before incubation (as described in chapter 3).

Immunotoxic effects have been described for several contaminants in the chicken embryo. The bursa of Fabricius is an important immune organ, in which B lymphocytes mature (Glick, 1983; Nikolaidis *et al.*, 1988b), and as such determines the immunocompetence of the early chick. A decrease in lymphocyte density has been described after exposure to PCBs (Brunström *et al.*, 1990b; Andersson *et al.*, 1991; Grasman and Whitacre., 2001), TCDD (Nikolaidis *et al.*, 1990), and hexabromobiphenyl (Vos and van Genderen, 1973). Eventually, a decreased lymphocyte density may compromise the newly hatched chick upon infectious challenges (Hoffman *et al.*, 1996; Grasman and Whitacre., 2001). Thus, the observed decrease in lymphocyte density again indicates the toxic potency of the contaminants present in the oystercatcher eggs. The concentrations of PCBs in the extracts were comparable to those from the mentioned studies. There is only limited information on interspecies differences for sensitivity towards effects on the bursa of Fabricius. When comparing studies on common tern chicks and american kestrels, it appears that wild bird species are similarly sensitive towards effects on the bursa of Fabricius (Hoffman *et al.*, 1996; Bosveld *et al.*, 2000). From the comparison between two other studies, the chicken appears to be more sensitive than the cormorant: decreased lymphocyte density was observed in the chicken at a dose of 0.13 ng/g egg PCB126 (Grasman and Whitacre, 2001), while it was only observed at a concentration of 11.7 ng/g egg TCDD in the cormorant (Powell *et al.*, 1998). As Andersson *et al.* (1991) showed that TCDD is 10 times more potent in inducing immunotoxic effects than PCB126, the chicken seems to be 900 times as sensitive to effects on the bursa. It is not known if the oystercatcher shows a similar difference in sensitivity as compared to the chicken as the cormorant. Therefore, no conclusions can be drawn on the actual effects in the oystercatchers living in the Zeehavenkanaal, but the extracts are in potential capable to induce effects on the bursa of Fabricius.

In summary, the present study was aimed at assessing the possible embryotoxic effects of contaminants present in oystercatcher eggs from the Zeehavenkanaal area. The bioassay indicated the toxic potency of the contaminants present in the oystercatcher eggs. However, contaminant concentrations in general, and in particular of hexachlorobenzene and PCBs were probably below the concentrations associated with toxic effects such as malformations and survival. In addition, from the bioassay in which the extract was tested, it can be concluded that effects on EROD activity and heme biosynthesis are unlikely to occur in this population. However, as effects on the immunocompetence of oystercatcher embryos or chicks could not be excluded, further studies on the actual effects in the population in the Zeehavenkanaal area are recommended.

Acknowledgements
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CHAPTER 7

THE PREDICTIVE VALUE OF EROD INDUCTION FOR TOXICITY IN THE CHICKEN EMBRYO

Submitted to Ecotoxicology

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Abstract
This study was aimed at establishing the predictive value of EROD induction in the chicken embryo for toxic effects on the population level. Therefore, a statistical analysis was performed on a dataset containing data on EROD induction, mortality and incidence of malformations, obtained from several experiments in which chicken embryos were exposed to contaminants with the capacity to bind to the Ah receptor from the start of development. The results supported the assumption that EROD activity and toxicity may be associated phenomena rather than causally related.

Introduction
In field studies, EROD induction in pipping embryos or chicks of various avian species has been used as a biomarker for contamination. EROD is an enzyme that belongs to the cytochrome P450 system. The induction of this system has been described to be the result of the binding of a compound to the Ah receptor (Mason *et al.*, 1985 and 1986; Safe, 1990). Compounds with a coplanar figuration, such as dioxins and coplanar PCBs, excert their toxic effects, at least in part, by binding to this receptor. However, a direct causative relation between EROD induction and toxicity has not been determined so far (Rifkind *et al.*, 1985, Custer *et al.*, 1999). Several field studies have shown a relation between EROD induction and toxic endpoints in wild bird species (Hoffman *et al.*, 1987 and 1993; Murk *et al.*, 1994; van den Berg *et al.*, 1994; Sanderson *et al.*, 1994), rats (Mason *et al.*, 1985 and 1986) and fish (van der Weiden *et al.*, 1992). In the bird studies mentioned, such a relation was mostly obtained from correlations between EROD activity and concentrations of complex mixtures of contaminants, compared to reference sites. Other studies on wild bird species indicate that EROD induction is not always associated with toxic effects (Bellward *et al.*, 1990; Rattner *et al.*, 1993, 1994 and 1997; Bosveld *et al.*, 1995; Elliott *et al.*, 1996; Custer *et al.*, 1999; Halbrook *et al.*, 1999). From these studies, it is evident that there is no clear cut relation between EROD induction in pipping embryos and toxic effects in bird populations. Therefore, in spite of all the studies made, the predictive value of EROD induction for possible toxic effects in the avian embryo remains to be resolved.
The present study was performed to establish the predictive value of EROD induction for possible toxic effects in the avian embryo. To this end, regression analyses were performed on data from several experiments in which chicken embryos were exposed to contaminants with the capacity to bind to the Ah receptor from the onset of incubation. This dataset comprised data on EROD induction, mortality (during the early stages, during the later stages and total), and abnormalities (both in live and dead embryos and total).

Next page: ***Table 1.*** *Database on EROD induction and toxicological parameters. Data are from different experiments, with each dose as an experimental unit. Total mortality (M), mortality in early stages (EM) and mortality in late stages (LM) are expressed in % of total fertilized eggs; total malformations (D) is expressed as % of total fertilized eggs, malformations among dead (DD) and malformations among living embryos (DL) are expressed as % of dead and living embryos, respectively. EROD induction is activity in exposed embryos divided by control activity. Contaminants include six fractions of whole body extracts from Atlantic Uria aalge (AU-I – AU-VI) and Baltic Uria aalge (BU-I – BU-VI), described by in chapter 5 and extracts from oystercatcher eggs from the Zeehaven canal in the Netherlands (ZO), described in chapter 6. For these extracts, doses are expressed as bird egg equivalents (beq).*

Table 1

Contaminant	dose	EROD induction	M	EM	LM	DD	DL	D
PCB126, µg/egg	0	1.0	20.0	6.7	13.3	33.3	0	6.7
PCB126, µg/egg	0.001	0.9	16.7	0	16.7	0	10.0	8.3
PCB126, µg/egg	0.012	1.0	26.7	20.0	6.7	0	0	0
PCB126, µg/egg	0.115	8.8	84.6	69.2	15.4	27.3	0	23.1
HCB, µg/egg	0	1.0	60.0	30.0	30.0	16.7	0	10.0
HCB, µg/egg	0.615	1.5	20.0	20.0	0	0	12.5	11.1
HCB, µg/egg	2.075	0.3	60.0	30.0	30.0	40.0	25.0	33.3
HCB, µg/egg	4.25	0.9	80.0	53.3	26.7	16.7	0	12.5
HCB, µg/egg	6.7	0.8	80.0	48.0	32.0	0	0	0
HCB, µg/egg	9.2	0.4	80.0	48.0	32.0	0	0	0
ZO, beq	0	1.0	10.0	10.0	0	0	0	0
ZO, beq	0.01	0.9	25.0	25.0	0	0	0	0
ZO, beq	0.1	0.6	50.0	25.0	50.0	50.0	50.0	50.0
ZO, beq	1	3.5	18.2	18.2	0	0	33.3	27.3
AU-I, beq	0	1.0	57.7	44.4	13.3	5.6	0	2.9
AU-I, beq	0.01	2.1	10.0	10.0	0	0	100.0	10.0
AU-I, beq	0.1	0.6	72.3	72.3	0	33.3	0	9.1
AU-I, beq	1	6.8	50.0	30.0	20.0	60.0	0	30.0
AU-II, beq	0.01	1.0	30.3	30.3	0	0	0	0
AU-II, beq	0.1	1.2	30.0	30.0	0	14.3	0	10.0
AU-II, beq	1	1.2	25.0	12.5	12.5	16.7	0	12.5
AU-III, beq	0.01	0.6	71.4	71.4	0	0	0	0
AU-III, beq	0.1	1.7	45.5	45.5	0	0	0	0
AU-III, beq	1	1.2	33.3	33.3	0	0	0	0
BU-I, beq	0.01	1.5	37.5	25.0	12.5	0	0	0
BU-I, beq	0.1	13.3	66.7	55.6	11.1	33.3	16.7	22.2
BU-I, beq	1	14.5	75.0	62.5	12.5	50.0	16.7	25.0
BU-II, beq	0.01	0.8	33.3	22.2	11.1	33.3	0	22.2
BU-II, beq	0.1	1.5	33.3	22.2	11.1	0	0	0
BU-II, beq	1	2.3	41.7	33.4	8.3	28.6	0	16.7
BU-III, beq	0.01	0.7	50.0	50.0	0	0	0	0
BU-III, beq	0.1	0.5	37.5	37.5	0	0	0	0
BU-III, beq	1	1.4	20.0	0	20.0	25.0	0	20.0
AU-IV, beq	0	1.0	30.4	23.4	7.0	5.6	0	2.9
AU-IV, beq	0.01	0.7	22.2	11.1	11.1	0	0	0
AU-IV, beq	0.1	1.7	14.3	14.3	0	0	0	0
AU-IV, beq	1	15.1	55.6	55.6	0	0	0	0
AU-IV, beq	0.01	0.8	12.5	12.5	0	0	0	0
AU-V, beq	0.1	0.3	57.1	57.1	0	33.3	0	14.3
AU-V, beq	1	8.2	85.7	42.9	42.9	200.0	0	28.6
AU-VI, beq	0.01	1.0	33.3	33.3	0	0	0	0
AU-VI, beq	0.1	0.5	30.0	30.0	0	28.6	0	20.0
AU-VI, beq	1	0.8	10.0	10.0	0	0	0	0
BU-IV, beq	0.01	1.3	33.3	33.3	0	0	0	0
BU-IV, beq	0.1	11.7	37.5	37.5	0	0	0	0
BU-IV, beq	1	6.7	90.0	70.0	20.0	300.0	0	21.4
BU-V, beq	0.01	1.0	14.3	14.3	0	33.3	0	20.0
BU-V, beq	0.1	0.8	0.0	0	0	0	0	0
BU-V, beq	1	1.6	37.5	37.5	0	0	0	0
BU-VI, beq	0.01	1.4	20.0	20.0	0	0	50.0	10.0
BU-VI, beq	0.1	1.0	40.0	20.0	20.0	0	0	0
BU-VI, beq	1	1.6	22.2	22.2	0	0	0	0
Aroclor, µg/egg	0	1.0	26.3	16.3	10.0	19.0	0	5.3
Aroclor, µg/egg	50	2.4	25.0	15.8	9.2	26.3	0	6.3

Materials and methods

Several experiments were performed, in which chicken embryos were exposed to increasing concentrations of contaminants. Contaminants included PCB126, hexachlorobenzene, Aroclor 1254, extracts from Baltic and Atlantic guillemots, and extracts from oystercatcher eggs from the Netherlands. These experiments have been described in detail elsewhere (chapters 4, 5, 6 and appendix). Data included in the statistical analysis were EROD induction, total mortality (%), mortality during the early stages, i.e. the first week of development (% of total mortality), mortality during the later stages, i.e. the last two weeks of development (% of total mortality), incidence of malformations (%), incidence of malformations among living embryos (%) and incidence of abnormalities among dead embryos (%). These data are summarized in table 1.

Statistics

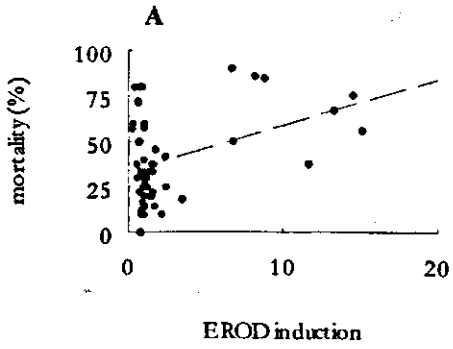
Statistical analysis was performed under genstat 5.5. Regression analysis was performed on mortality and abnormality data using EROD induction as the descriptive variable. All analyses were performed on raw data, and the significance level was stated as 5%.

Results

Regression analysis on data on EROD induction, mortality and malformations observed in treatment groups revealed that EROD correlated significantly with the toxicological parameters included (table 2). Although regressions were significant for EROD and total mortality ($p=0.003$) or mortality during the early stages ($p=0.001$), only 14 or 17% of the variance was explained by EROD induction, respectively (figure 1). The occurrence of malformations correlated significantly with EROD induction, but EROD only explained 6% of the variance for malformations among all embryos ($p=0.042$) and 6% for malformations among dead embryos ($p=0.036$) (figure 2). Toxicological parameters correlated significantly with each other: malformations among all embryos explained 28% of the variance in mortality among late embryos ($p<0.001$), while malformations among dead embryos explained 21% of the variance in total mortality.

Table 2. Correlation matrix between EROD induction, occurrence of malformations (%) and mortality (%). Values in brackets are p-values

	EROD induction	Total malformations	Malformations among dead embryos
Total malformations	0.057 (0.042)		
Malformations among dead	0.062 (0.036)		
Malformations among living	- (0.748)		
Total mortality	0.138 (0.003)	0.074 (0.024)	0.213 (<0.001)
Mortality in early stages	0.165 (0.001)		
Mortality in late stages	- (0.641)	0.275 (<0.001)	0.163 (0.001)



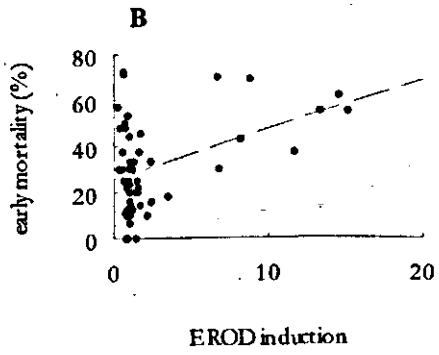


Figure 1. Correlations between EROD induction and total mortality (A) and mortality during the early stages (B). Curves reflect regression lines (A: p=0.003; B: p=0.001).

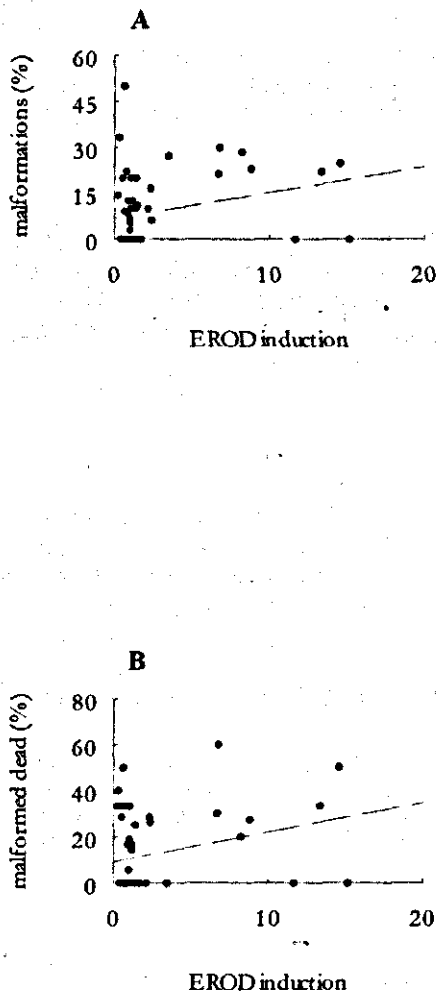


Figure 2. *Correlations between EROD induction and incidence of malformations among all embryos (A) or among dead embryos (B). Curves reflect regression lines (A: p=0.042; B: p=0.016)*

Discussion

The statistical analysis performed on data of EROD induction and incidences of mortality and malformations, revealed significant correlations between EROD induction and these parameters of toxicity. However, only part of the variance observed in toxicological parameters included was explained by EROD induction, implying that other factors must be contributing to toxicity. These results support the study by Rifkind *et al.* (1985), who found that induction of EROD and toxicity were not causally related; they suggested that the phenomena may be associated, but causally unrelated, or they may be causally related, but with an event secondary to induction, and differentially modified by different contaminants. In either situation, biochemical events other than the induction of the mixed function oxidase system must mediate toxicity.

The finding that EROD induction only explained a small part of the observed toxicity may be the result of the fact that EROD induction can only be measured in living embryos, whereas the toxic endpoints mainly reflect the dead embryos. Higher levels of explanation are expected when both parameters are measured in the same individuals. In addition, other toxicological endpoints such as hormonal disturbances or immunological parameters, were not included in the analysis, although these parameters have been described to be correlated with EROD induction in wild bird species. For instance, Murk *et al.* (1994) and van den Berg *et al.* (1994) found significant correlations between EROD induction and thyroid hormone parameters and plasma retinol and retinyl levels in common terns and cormorants. From the present study, it cannot be excluded that such sublethal parameters may be better explained by EROD induction. The finding that EROD induction was correlated with survival and incidence of malformations was in line with several field studies. In common terns, a 77% increase in AHH activity (which is, like EROD, also a CYP450 1A1 associated activity, Rifkind *et al.*, 1994), was found to be co-existant with decreased egg weight, embryo weight and femur length/body weight ratio (Hoffman *et al.*, 1993). In accordance, a three fold induction of EROD activity in Forster's tern was related to decreased hatching success, decreased hatchling weight and decreased femur length, as well as with the increased incidence of abnormalities (Hoffman *et al.*, 1987). Also in great blue herons, induction of EROD activity was found to be correlated with an increase in abnormalities among chicks, and to several growth parameters (Sanderson *et al.*, 1994). Even though EROD induction has been shown to be associated with increased risk, some studies reported EROD induction without concurrent toxic effects (Bellward *et al.*, 1990; Rattner *et al.*, 1993, 1994 and 1997; Elliott *et al.*, 1996; Halbrook *et al.*, 1999). Custer *et al.* (1999) found correlations between EROD activity and PCB concentrations in double-crested cormorants, while none of the affected parameters for reproductive output were correlated with EROD. Another study showed that, although delayed hatching was observed in common tern embryos that had higher PHAH levels, which were in turn related to EROD activity, no effects were observed on hatching success, morphology, or embryonal respiration (Bosveld *et al.*, 1995). The reason for the lack of concurrent toxic effects in these studies is unclear, but may be based on the species' differing sensitivities, different contaminant composition, differing concentrations or even undetected toxic effects; the disappearance of eggs has since long been recognized as a biasing factor (Ratcliffe, 1970).

The aim of the present study was to establish the predictive value of EROD induction for possible adverse effects on the population level. It was shown that EROD induction and toxicity are associated, but causally unrelated phenomena, as suggested

by Rifkind *et al.* (1985). Considering field studies in which EROD induction was observed without concomittant toxic effects, identification of a possible risk for toxic effects solely dependent on induced EROD activity must be considered as a conservative approach that will not underestimate the actual risk.

Acknowledgements
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CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

In this thesis, several studies on the effects of persistent organic pollutants on the developing chicken embryo are described. The main objectives of the research were twofold. Firstly, the hypothesis that contaminant induced thiamine deficiency plays a role in avian embryotoxicity was tested. Secondly, the current contamination pressure in the Baltic guillemot was assessed. This was also studied for the oystercatcher foraging in the Zeehavenkanaal area in the Netherlands. The research was performed using the chicken embryo as a model species. It should be noted that the use of a model organism for the estimation of risks in other species is an elegant, though complicated, measuring tool. The chicken embryo is often chosen as a model species, as it has been shown to respond in a sensitive way to the toxic action of contaminants. Whether results obtained in this model can be translated to the field situation will always be a point of discussion: different species will show different responses. However, the chicken embryo is an indicator species that can be used to determine the toxic potencies of chemicals, present in the field situation, and is therefore considered a valuable tool in risk estimations.

Chapter 1 gives the background of the aims of the research. In addition, it provides an introduction to the biomarkers used in the studies. *Chapter 2* describes the development of an optimized bioassay. This bioassay was adapted to the requirements for detection of potential effects on thiamine status, as measured from transketolase activity. To this end, a novel carrier was constructed from egg yolk lipids. Later, it was observed that this carrier may be a facilitating factor for the spread of infection among eggs, and therefore it was only used in the studies with a focus on contaminant induced thiamine deficiency. In *chapter 3*, the validation of the bioassay is described. It is shown that the concentration of PCBs in the embryo is constant during development and can be predicted from the injection dose. Model compounds were tested for their potency to induce thiamine deficiency in *chapter 4*. Although furazolidone (an antibiotic that was used as a positive control) decreased transketolase activity, none of the tested POPs affected this enzyme, indicating that they did not deplete thiamine levels in the chicken embryo.
Chapters 5 and *6* describe the toxic effects of whole body extracts of common guillemots and of egg extracts of oystercatchers in the chicken embryo, respectively. Both species were chosen for study as they lived in contaminated areas. The guillemot extract increased the incidence of malformations and decreased survival. Both extracts induced EROD activity in the livers of embryos exposed to realistic egg concentrations. In addition, both extracts reduced lymphocyte density in the bursa of Fabricius, at rather low contaminant levels, indicating that immunotoxic effects may be of importance in embryotoxicity. For both species, it was concluded that toxic effects cannot be excluded under the present contaminant levels. The guillemot

extracts were also tested for their potency to induce thiamine deficiency. None of the thiamine dependent biomarkers were affected, indicating that thiamine depletion was not induced by the present contaminants. Based on the results of experiments conducted with model compounds (*chapter 4*) or whole body guillemot extracts (*chapter 5*), it can be concluded that contaminant induced thiamine deficiency probably does not play a role in observed embryotoxicity.
EROD induction was found to be a sensitive biomarker in the chicken embryo, responding at contaminant levels that were comparable to those in eggs in the field situation. Its predictive value for toxicity in birds was assessed in *chapter 7*. In this study, it was demonstrated that EROD induction is associated with toxicity, but that the two phenomena are causally unrelated.

Concluding remarks
The most important results from the studies described lead to a threefold conclusion.

Firstly, no evidence was found for the hypothesis that contaminant induced thiamine deficiency plays a role in avian embryotoxicity. This is in line with recent studies in fish, which indicate that thiamine deficiency plays a role in observed embryo mortalities, but that this is not associated with contamination. Fitzsimons *et al.* (1995) described the incidence of early mortality in Lake Ontario lake trout, which was independent of contaminant levels Palace *et al.* (1998) found that these fish displayed elevated oxidative stress indices, which was not correlated with early mortality among their offspring. In line with these studies, Asplund *et al.* (1998) and Honeyfield *et al.* (1998b) reported that levels of organohalogens were not different between healthy fry and fry suffering from the early mortality syndrome or their respective mothers. Finally, it has been shown that increased NADPH consumption in fish did not lead to thiamine depletion through stimulation of the pentose phosphate pathway (Åkerman *et al.*, 2001a and b; Tjärnlund *et al.*, 2001). In addition, bird embryos may be prevented from contaminant induced thiamine deficiency if the protein bound thiamine pool, which is deposited in the egg yolk by the mother bird, is large enough. This pool is a reflection of the diet of the mother bird (Naber, 1979; Adiga and Ramana Murty, 1983), and has been shown to determine the thiamine status in her progeny (Olkowski and Classen, 1999). Therefore, contaminant induced thiamine deficiency does not seem to play a role in embryotoxicity in birds.

Secondly, EROD induction was found to be associated, but causally unrelated to toxicity. EROD induction explained about 20% of observed toxicity in chicken embryos, but has also been observed in birds without concurrent toxic effects. Therefore, EROD induction must be considered as a biomarker for exposure to contaminants with the capacity to bind to the Ah receptor rather than as a direct predictor for toxicity.

Thirdly, observations on the bursa of Fabricius indicated that contaminants present in birds at this moment may have immunotoxic consequences. This biomarker was observed to be very sensitive, responding to contaminant concentrations which were lower than those currently present in field situations. These results indicate the need for further research on the role of impairment of the immune function in avian embryotoxicity.

APPENDIX

INDUCTION OF EROD ACTIVITY THROUGHOUT EMBRYONAL DEVELOPMENT OF THE CHICKEN

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Abstract
EROD induction is often used as a biomarker for contamination. It has been demonstrated to be related to toxic endpoints. In field studies, EROD induction levels in pipping bird embryos are generally lower than those measured in laboratory studies. The present study was aimed at testing the hypothesis that toxic events leading to mortality before the end of incubation would cause the death of individuals with higher EROD induction levels, resulting in an apparently decreased average EROD induction at the end of development. To this end, chicken embryos were exposed to Aroclor 1254 from the onset of incubation, and EROD activity was measured during the early and later stages in exposed and control embryos. However, no toxic effects were observed, probably as a result of a too low concentration of Aroclor. Therefore, the hypothesis could not be tested. It is shown that EROD induction in embryos exposed throughout development correlated with the inducibility of the enzyme system. In order to test the hypothesis, the study should be repeated with higher concentrations of PCBs.

Introduction
In field studies, EROD induction in pipping embryos or chicks of various avian species has been used as a biomarker for contamination. In a previous study, it was shown that EROD induction is associated with toxicity (chapter 7). However, the vast amount of literature at the subject shows that in field situations, EROD induction shows high variability among and within species, but rarely exceeds the factor of 5-8 times the control level as found in selected reference sites (table 1). The observed variation may partly arise from species differences in sensitivity towards EROD induction (Walker *et al.*, 1984; Bosveld, 1995; Fossi *et al.*, 1995; Sanderson and Bellward, 1995; Kennedy *et al.*, 1996; Davis *et al.*, 1997; Henriksen *et al.*, 2000), and partly from the variation in contaminants and concentrations encountered. In the laboratory, where embryos are exposed at later developmental stages, predominantly high induction levels are found (table 1). The only bird species that has been found to display up to 80 times induced EROD activities in a comparative field study, is, to our knowledge, the black crowned night heron on Cat Island, Green Bay, Michigan (Rattner *et al.*, 1993 and 1994), but this may be explained by the exceptionally low EROD activity found in reference sites (Bosveld and van den Berg, 1994). Although interspecies differences explain part of the variation, there is a clear difference when comparing laboratory studies with field studies performed for the same species (table 1). Apart from differences in contaminant concentrations, laboratory studies differ from field studies in exposure regime. In the field situation, exposure starts at the onset of incubation, whereas in the laboratory this does not necessarily have to be the case. The differences in induction levels observed as a consequence of different exposure regimes may result from several causes. Biotransformation of the contaminants may lead to a decreased exposure at the end of development, when the embryo is exposed from the onset of incubation. Chicken embryos have been described to be able to metabolize some PCBs from day 5-7 in development (Brunström *et al.*, 1982; Hamilton *et al.*, 1983; Brunström, 1986), and the chorioallantoic membrane and the yolk have metabolic capacities at even earlier stages of development (Heinrich-Hirsch *et al.*, 1990; Annas *et al.*, 1999 and 2000). However, most PCB congeners occurring as contaminants in the environment are generally resilient towards metabolic processes, and the elimination efficiency by biotransformation of PCBs is believed to be rather low (Hamilton *et al.*, 1983; Wehler

Table 1. *Literature overview. T_0, start of exposure (day); T_e, end of exposure (day of measurement); H=hatching stage; F, field study; L, laboratory study. Induction= ratio induced/control EROD activity.*

reference	F/L	species	contaminants	T_0	T_e	concentration	induction
Bosveld et al., 1992	L	chicken	TCDD	17	18	70 ng/egg	100
Bosveld et al., 1992	L	chicken	PCB126	17	18	7000 ng/egg	80
Brunström & Lund, 1988	L	chicken	PCB77	5	7	16 µg/kg	20
Brunström & Andersson, 1988	L	chicken	PCB77	7	9	1450 pmol/kg egg	36.4
Brunström & Andersson, 1988	L	chicken	PCB126	7	9	11000 pmol/kg egg	29.5
Brunström, 1992	L	chicken	PCB77	7	10	1 µg/kg egg	7.6
Brunström, 1992	L	chicken	PCB126	7	10	0.05 µg/kg egg	9.6
Brunström, 1992	L	chicken	PCB77	9	10	1 µg/kg egg	8.4
Brunström, 1992	L	chicken	PCB126	9	10	0.05 µg/kg egg	5.4
Sanderson & Bellward, 1995	L	chicken	TCDD	16	H	1 µg/kg egg	56
Engwall et al., 1994	L	chicken	PCNs	7	10	1 mg/kg	6
Engwall et al., 1994	L	chicken	Halowax	4	18	1 mg/kg	2
Engwall et al., 1994	L	chicken	HpCN mixture	4	18	1 mg/kg	1
Engwall et al., 1994	L	chicken	HxCN mixture	4	18	1 mg/kg	2
Hoffman et al., 1998	L	chicken	PCB126	4	H	0.3 ng/g	16.8
Hoffman et al., 1998	L	chicken	PCB77	4	H	1.2 ng/g	4.4
Brunström & Lund, 1988	L	turkey	PCB77	7	9	631 µg/kg	5.6
Engwall et al., 1994	L	eider duck	Halowax	5	24	1 mg/kg	4
Engwall et al., 1994	L	eider duck	HpCN mixture	5	24	1 mg/kg	3.5
Engwall et al., 1994	L	eider duck	HxCN mixture	5	24	1 mg/kg	11
Bosveld et al., 1995	F	common tern	PCBs	0	H	117 µg/g	4
Hoffman et al., 1998	L	common tern	PCB126	4	H	434 ng/g	20.3
Hoffman et al., 1993	L	common tern	PCBs	0	H	7.6 µg/g	2
van den Berg et al., 1994	F	cormorant	PCBs	0	H	1372 µg/g lipid	1.3
Davis et al., 1997	F	cormorant	unknown	0	H		4-8
Sanderson & Bellward, 1995	L	double-crested cormorant	TCDD	22	H	100 µg/kg egg	84.6
Powell et al., 1998	L	double-crested cormorant	PCB126	0	H	698 µg/kg egg	9.6
Powell et al., 1998	L	double-crested cormorant	TCDD	0	H	10.7 µg/kg egg	7.9
Rattner et al., 1997	F	black-crowned night heron	mixture	0	H	2.6-3.4 µg/g	6-9
Rattner et al., 1994	F	black-crowned night heron	PCBs	0	H	10 µg/g	85
Rattner et al., 1993	F	black-crowned night heron	PCBs	0	H	10 µg/g	85
Halbrook et al., 1999	F	great blue heron	PCBs	0	H	0.86 µg/g	0
Halbrook et al., 1999	F	great blue heron	PCBs	0	H	2.01 µg/g	0
Sanderson et al., 1994	F	great blue heron	dioxin/PCBs	0	H	42 ng/kg dioxins	3.2
Sanderson et al., 1994	F	great blue heron	dioxin/PCBs	0	H	10 ng/kg dioxins	1.2
Sanderson et al., 1994	F	great blue heron	dioxin/PCBs	0	H	16 ng/kg dioxins	2.7
Sanderson et al., 1994	F	great blue heron	dioxin/PCBs	0	H	211 ng/kg dioxins	5.5
Sanderson & Bellward, 1995	L	great blue heron	TCDD	22	H	100 µg/kg egg	566.7
Bellward et al., 1990	F	great blue heron	PCBs	0	H	2.1-4.3 µg/g	1.7-2.6
Ellenton et al., 1985	F	herring gull	dioxin/PCBs	0	20	86/64 pg/g µg/g	5
Ellenton et al., 1985	F	herring gull	dioxin/PCBs	0	25	86/64 pg/g µg/g	9
Boersma et al., 1986	F	herring gull	PCBs	0	25	50 µg/g	2
Hoffman et al., 1998	L	american kestrel	PCB126	4	H	233 ng/g	11.5
Hoffman et al., 1998	L	american kestrel	PCB77	4	H	1000 ng/g	2.4

et al., 1990; Brunström, 1992; van den Brink and Bosveld, 2001). A second explanation is hypothesised here, and states that the average EROD induction level in a population of embryos exposed from the start of incubation may be reduced at the time of hatching as a consequence of lethal toxic effects that occur during development. In previous experiments, in which chicken embryos were exposed to PCBs from the onset of incubation, mortality rates as compared to control groups were rather high. This is in line with many field data, in which mortality among contaminated embryos was enhanced over background levels (e.g. described in reviews by Gilbertson *et al.*, 1991; Grasman *et al.*, 1998), as well as by laboratory data (Walters *et al.*, 1987; Brunström and Lund, 1988; Brunström *et al.*, 1991 and 1992; Brunström, 1992; Nosek *et al.*, 1993; Gustafsson *et al.*, 1994). In addition, Elliott *et al.* (1988) reported higher levels of several organochlorine compounds in failed gannet eggs when compared to fresh eggs. In accordance with this, Nisbet and Reynolds (1984) and Becker *et al.* (1993) found higher levels of PCBs in unhatched common tern eggs when compared to concentrations in successfull eggs, and Thyen *et al.* (2000) showed that concentrations of hexachlorobenzene and hexachlorocyclohexane were higher in non-developed eggs of little terns than in developed eggs. Hence, we assume that the relatively low average induction levels of EROD activity observed at the end of the embryonal development result from the dying of embryos with higher induction levels. If the hypothesis proves to be true, this could have implications for monitoring programmes, as risk estimates are mostly based on data obtained from pipping embryos or freshly hatched chicks, which then represent an underestimation of the situation on the population level.
The aim of the present study was to test this hypothesis. Chicken embryos were exposed to Aroclor 1254 from the onset of incubation. The days of measurement were based on previous observations, which indicated that mortality was mostly increased during the early stages of development (days 5-9); days for measurements at later developmental stages were chosen to be comparable to other studies, in which pipping embryos or freshly hatched chicks are used. However, the hypothesis could not be tested, as no toxic effects were observed. Nevertheless, a descritpion is given here of the development of EROD induction and its relation to enzyme inducibility.

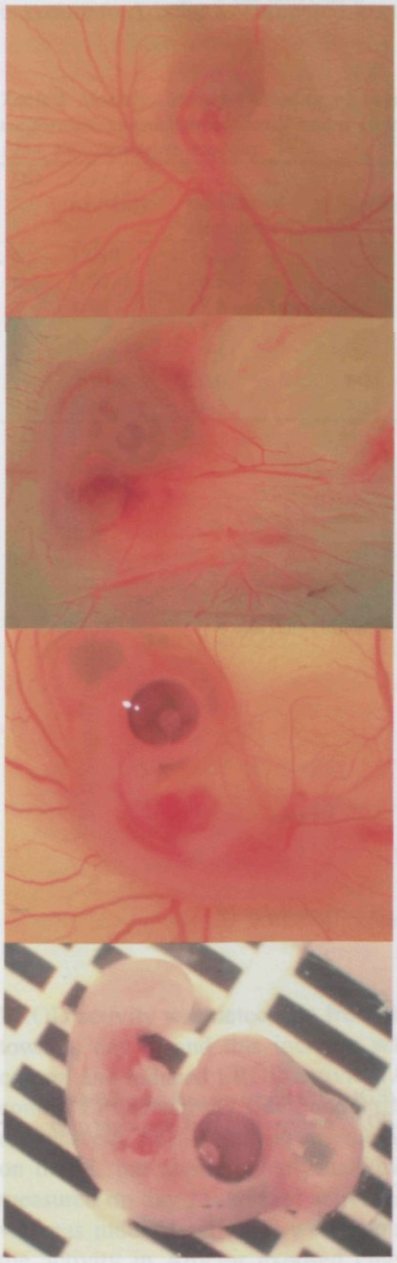
Materials and Methods

Fertilized chicken eggs were kindly provided by Verbeek's poultry farm (Lunteren). They were injected with Aroclor 1254 (0 or 50 µg/egg) prior to incubation as described before (chapter 2), with sterilised propylene glycol as a carrier. On days 5-9 embryos were dissected from the yolk sac, examined for gross abnormalities under the microscope and frozen in liquid nitrogen. They were stored at -80°C until further processing. On day 19, embryos were decapitated and examined for gross abnormalities. Their livers were frozen in liquid nitrogen and stored at -80°C. In figure 1, representative pictures of the different embryonal stages under study are shown. Freshly hatched chicks were aneasthetized using diethylether before decapitation. Their livers were processed similarly as those of 19 day old embryos. EROD activity was measured in whole embryos or livers as described before (chapter 5).

Statistics

Statistical analysis was performed under genstat 5.5. For the development of EROD induction, ANOVA was used to detect differences in EROD activity between days

(within groups) and between groups (within days). Regression analysis was performed on the EROD activities and EROD inductions using developmental day as a descriptive variable. Regression analysis was performed on the EROD induction data using data from Hamilton *et al.* (1983) as the descriptive variable.



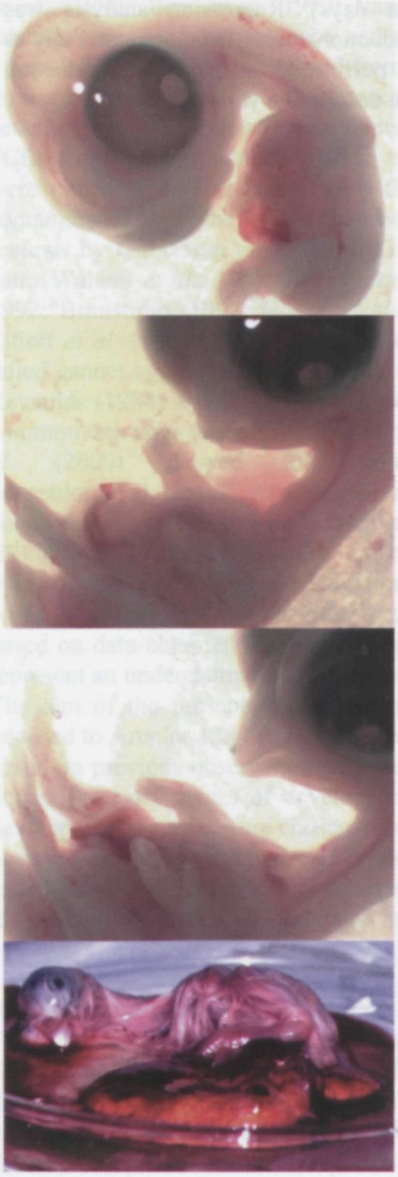
Embryonal day 3

Embryonal day 4

Embryonal day 5

Embryonal day 6

Figure 1



Embryonal day 7

Embryonal day 8

Embryonal day 9

Embryonal day 19

Figure 1. *Embryonal stages under study. Embryonal days (ED) 4-5: embryo on yolk sac; ED 6-9: embryo detached from yolk sac (ED 6: on histology grid); ED19: embryo with yolk sac, right after opening of the egg.*

Results

Embryos from the studied developmental stages are shown in figure 1. Total mortality was not increased after exposure to Aroclor (table 2). Malformations were only observed in dead embryos. Although there was no difference in total occurrence of malformations, one malformed embryo was observed in an earlier stage after exposure to Aroclor as compared to controls (table 2).

Table 2. *Mortality (dead/fertilized eggs), cumulative mortality (%) and appearance of malformations (malformed/dead) among control embryos and embryos exposed to Aroclor 1254. DD, developmental day.*

DD	*control* mortality	cumulative mortality (%)	malformations	*Aroclor* mortality	cumulative mortality (%)	malformations
5	12/80	15.0		12/76	15.7	
6	1/80	16.3			15.7	
7	1/80	17.5		1/76	17.1	
8		17.5			17.1	1/19
9		17.5		1/76	18.4	
19	2/80	20.0	1/21	4/76	23.7	
21	5/80	26.3	3/21	1/76	25.0	4/19

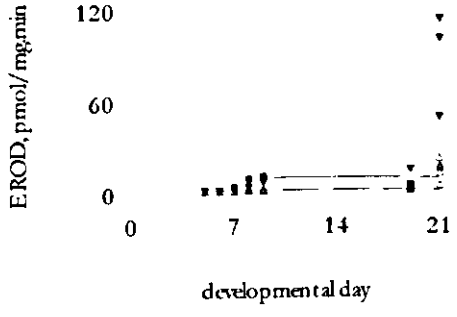


Figure 2. *EROD activity in control (open symbols) and Aroclor exposed (filled symbols) embryos on different developmental days. Curve fits were based on EROD activities on days 5 through 9, and extrapolated until hatching. Solid line, Aroclor; dashed line: control.*

EROD activity was detectable from day 5 of development (figure 2). It increased fast towards day 9, and this increase was best described by a logistic curve, both for control and induced EROD activity (Aroclor: $p<0.001$; control: $p=0.003$). On day 19, control EROD activity was 2 and 3 times the activity on days 8 and 9 of development, respectively. For Aroclor exposed embryos, the activity on day 19 was similar to that on day 8, but approximately half the activity on day 9. Maximum activities were measured on day 21: control activities were 7 times the activity measured on day 19, whereas induced activities were about 20 times the activity on day 19. On this day, the activity in Aroclor exposed embryos was significantly higher than that in the control embryos ($p<0.001$). Development of EROD induction is shown in figure 3. From this figure, it is clear that induction over control levels increases drastically from day 5 to day 9; this increase could be described by an exponential curve

(induction=$0.40*1.47^{\text{developmental day}}$-1.21; $p<0.001$). Induction levels were lower on days 19 and 21. ANOVA analysis on the induction levels revealed that EROD induction on day 19 was similar to the induction on all days from day 5 onwards except for days 7 and 9, on which induction levels were significantly higher ($p<0.001$). On day 21, the induction level was similar to the induction on all days from day 6 onwards except for day 9, on which induction was significantly higher ($p<0.001$). Regression analysis of EROD induction versus enzyme inducibility (data from Hamilton *et al.*, 1983) revealed that inducibility was a good descriptive variable for EROD induction ($p=0.014$; 67.2% of variance explained, figure 4).

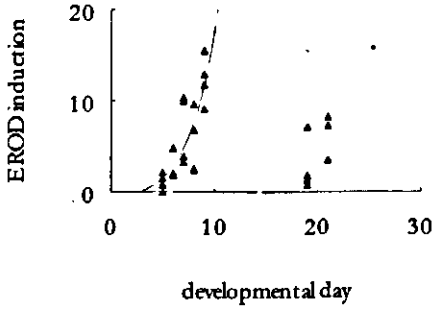


***Figure 3.** EROD induction throughout development. Dashed line is curve fit for days 5-9 (induction=$0.40*1.47^{X}$-1.21; $p<0.001$).*

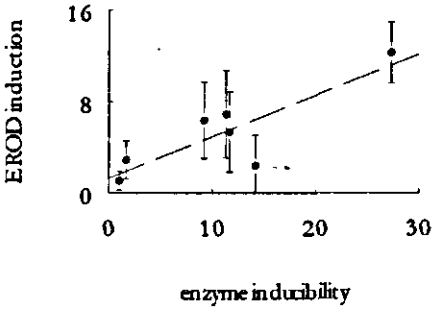


***Figure 4.** EROD induction in the developing embryo as function of enzyme inducibility (data from Hamilton et al., 1983). Values represent mean ± standard deviation; regression line represents the correlation between induction and inducibility ($p=0.014$) and explains 67.2% of the variance in EROD induction.p*

Discussion

Development of EROD activity throughout embryonal development showed great similarities to the data published by Hamilton *et al.* (1983), Hamilton and Bloom (1983) and Denison *et al.* (1986). However, the decrease in EROD induction on day 19 as compared to day 9 had not been described by these authors. Denison *et al.* (1986) found that induction factors on days 9 and 19 were comparable to each other, whereas the induction factor was decreased at day 19 as compared to day 9 in the study of Hamilton *et al.* (1983). The discrepancy between the study of Denison *et al.* (1986) and the results from the present study and those presented by Hamilton *et al.* (1983) may in part be explained by the matrix in which EROD activity was measured on day 9; whereas in the present study and the one by Hamilton *et al.* (1983) the whole body was used, Denison *et al.* (1986) only used the liver: from the study by Hamilton *et al.* (1983), it is clear that induction in the liver is only half that in the entire embryo (measured on day 10). Both Hamilton *et al.* (1983) and Denison *et al.* (1986) described a further decrease in the induction factor from day 19 to day 21. This was also found by Bosveld *et al.* (1997), who measured the inducibility of EROD activity in primary chicken embryo hepatocytes. In the present study, the induction factor on day 21 was not significantly different from the induction factor on day 19. This discrepancy may be due to variation; both in our study and those described in literature, the induction rates do not differ greatly between days 19 and 21.

The present study was performed to test the hypothesis that mortality due to toxic action of the contaminants early during development would lead to the selection of embryos with low EROD induction levels. However, no toxic effects were observed after exposure to Aroclor, probably as a result of a too low exposure concentration. Therefore, this hypothesis could not be tested. A regression analysis was performed on the EROD induction data, using the data from Hamilton *et al.* (1983) as the descriptive variable. This study was chosen as it was most comparable to the present data, because enzyme activities were measured in the same matrices. There was a significant relationship between the two parameters; inducibility of the enzyme system explained 67.2% of the variance in EROD induction after exposure from day 0, and EROD induction on day 21 could be explained very well by inducibility (calculated from the relative change as described by Hamilton *et al.*, 1983).

In conclusion, the results from present study could not be used to test the hypothesis that toxicity determines the final outcome of EROD measurements in the pipping chicken embryo. However, AHH inducibility was shown to be an important explaining factor for EROD induction. Further research, in which different, higher concentrations of PCBs are used, is needed to elucidate the influence of toxicity on measured average EROD induction in a population of exposed embryos.

Acknowledgements

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SAMENVATTING

In dit proefschrift worden verschillende studies naar de effecten van persistente organische verbindingen (POVs) op het zich ontwikkelende kippenembryo beschreven. De doelstellingen van het onderzoek waren tweeledig. Het eerste doel was te onderzoeken of een vermindering van de beschikbare hoeveelheid thiamine (vitamine B1) als gevolg van blootstelling aan contaminanten (zoals PCBs, pesticiden) een rol kan spelen bij waargenomen schadelijke effecten in vogelembryo's. Het tweede doel was vast te stellen of zeekoeten in de Baltische zee onder de huidige blootstellingsniveaus schadelijke effecten van aanwezige contaminanten kunnen ondervinden. Dit werd ook onderzocht voor scholeksters die leven bij het Zeehavenkanaal in Nederland. Voor het onderzoek werd het kippenembryo als modelsoort gebruikt. De toepassing van een modelorganisme voor de schatting van risico's in een andere soort is een elegante, maar gecompliceerde methode. Het kippenembryo wordt vaak als modelsoort gebruikt, omdat het gevoelig reageert op de toxische werking van contaminanten. De mate waarin resultaten van dit model naar de veldsituatie kunnen worden geëxtrapoleerd, zal altijd punt van discussie blijven: verschillende soorten zullen verschillend op stoffen reageren. Toch is het kippenembryo een indicatorsoort die kan worden gebruikt om mogelijke effecten van stoffen, die in het veld aanwezig zijn, aan te tonen. Daarom wordt het kippenembryo gezien als een waardevol systeem in de risicoschatting.

Hoofdstuk 1 geeft de achtergrond van de doelstellingen van het onderzoek. Daarnaast geeft het een introductie voor de biomarkers die in het onderzoek zijn gebruikt. *Hoofdstuk 2* beschrijft de ontwikkeling van een geoptimaliseerd bioassay (een biologisch testsysteem). Dit bioassay werd zodanig aangepast, dat het geschikt was om eventuele effecten van contaminanten op de thiaminehuishouding te signaleren, zoals gemeten uit de activiteit van transketolase. Dit enzym is afhankelijk van thiamine en zijn activiteit zal daarom afnemen als er minder thiamine beschikbaar is. Voor injectie van stoffen in het ei werd een nieuwe drager ontwikkeld, die werd verkregen uit dooiervetten. Later bleek dat deze drager de verspreiding van infecties tussen eieren zou kunnen vergemakkelijken, en daarom werd hij alleen toegepast in experimenten waarbij gekeken werd naar effecten van stoffen op de thiaminehuishouding. In *hoofdstuk 3* wordt een beschrijving gegeven van de verdeling van geïnjecteerde stoffen vanuit de dooier naar het embryo. De concentratie van PCBs in het embryo bleek gedurende de embryonale ontwikkeling constant te zijn, en bovendien te voorspellen uit de geïnjecteerde dosis. De effecten van modelstoffen op de activiteit van transketolase worden beschreven in *hoofdstuk 4*. Hoewel furazolidon (een antibioticum dat als positieve controle werd gebruikt) de activiteit van dit enzym verlaagde, bleek geen van de geselecteerde POVs dat te doen, hetgeen erop wijst dat ze niet leidden tot de vermindering van de beschikbare hoeveelheid thiamine in het kippenembryo.

In *hoofdstukken 5* en *6* worden studies beschreven waarin toxische effecten van extracten worden getest. De extracten werden verkregen uit Baltische en Atlantische zeekoeten en eieren van scholeksters van het Zeehavenkanaal in Nederland; beide soorten werden bestudeerd om het risico op schadelijke effecten van de bestaande vervuiling in de vogels te schatten. Het zeekoetextract verhoogde de frequentie van misvormde embryo's en verlaagde de overleving. Beide extracten induceerden de activiteit van EROD (een enzym dat erop wijst dat er dioxineachtige stoffen aanwezig zijn) bij doseringen die overeenkwamen met blootstellingsniveaus in eieren in het veld. Bovendien leidden beide extracten tot een relatieve verlaging van de lymfocytendichtheid in de bursa van Fabricius, het immuunorgaan waarin de B cellen rijpen. Dit werd waargenomen bij lage concentraties van de aanwezige contaminanten, hetgeen erop wijst dat immunotoxische effecten van belang kunnen zijn in embryotoxiciteit. Voor beide soorten werd geconcludeerd dat schadelijke effecten van contaminanten onder de huidige blootstellingsniveaus niet zijn uit te sluiten. De zeekoetextracten werden voorts getest op hun potentie om verlaging van de beschikbare hoeveelheid thiamine te veroorzaken. Geen van de thiamineafhankelijke biomarkers gaf een respons, hetgeen erop duidt dat blootstelling aan de aanwezige contaminanten niet resulteerde in thiaminedeficiëntie. Op basis van de resultaten van experimenten met modelstoffen (*hoofdstuk 4*) en met zeekoetextracten (*hoofdstuk 5*), kan geconcludeerd worden dat door contaminanten geïnduceerde thiaminedeficiëntie geen rol speelt bij de waargenomen embryotoxische effecten in het kippenembryo.
De inductie van EROD activiteit bleek in het kippenembryo een gevoelige biomarker te zijn, die reageerde op contaminantenniveaus die ook in het veld in eieren aangetroffen worden. De voorspellende waarde van deze biomarker voor toxische effecten is beschreven in *hoofdstuk 7*. Die studie geeft aan dat EROD inductie en toxiciteit in het vogelembryo causaal niet gerelateerd, maar wel onderling geassocieerd zijn.

Conclusies
De belangrijkste resultaten van de beschreven studies leiden tot een drieledige conclusie.

Ten eerste werd geen bewijs gevonden voor de hypothese dat door contaminanten geïnduceerde thiaminedeficiëntie een rol speelt in embryotoxiciteit bij vogels. Dit is in overeenstemming met recente studies in vissen, die aangeven dat thiaminedeficiëntie wel een rol speelt bij de waargenomen sterfte in vroege stadia, maar dat dit niet het gevolg is van blootstelling aan contaminanten. Fitzsimons *et al.* (1995) beschrijft het voorkomen van vroege sterfte in forellen in het Ontariomeer, zonder dat dat gerelateerd is aan contaminantenniveaus. Palace *et al.* (1998) vonden in deze vissen aanwijzingen voor oxidatieve stress, en ook dat was niet gecorreleerd met vroege sterfte. In overeenstemming hiermee toonden Asplund *et al.* (1998) en Honeyfield *et al.* (1998b) aan dat contaminantenniveaus niet verschilden tussen gezonde larven en stervende larven, of hun respectievelijke moeders. Inmiddels is aangetoond dat een verhoogde consumptie van NADPH (een vorm van energie in de cel) in vissen niet leidde tot een vermindering van de beschikbare hoeveelheid thiamine (Åkerman *et al.*, 2001a en 2001b; Tjärnlund *et al.*, 2001). Bovendien is het mogelijk dat vogelembryo's tegen door contaminanten geïnduceerde thiaminedeficiëntie worden beschermd als de thiaminevoorraad in de dooier hoog

genoeg is. Thiamine wordt door de moedervogel aan eiwit gebonden in de dooier opgeslagen, en is zodoende een weerspiegeling van haar dieet (Naber, 1979; Adiga en Raman Murty, 1983). Deze maternale voorraad van thiamine bepaalt de thiaminestatus van haar nakomelingen (Olkowski en Classen, 1999). Daarom kan geconcludeerd worden dat thiaminedeficiëntie als gevolg van blootstelling aan contaminanten waarschijnlijk geen rol speelt in embryotoxiciteit in vogels.

Ten tweede bleek EROD inductie geassocieerd te zijn met toxiciteit, maar zonder een causaal verband. De inductie van de EROD activiteit verklaarde ongeveer 20% van de waargenomen toxiciteit in het kippenembryo. Bovendien is EROD inductie ook beschreven in vogels zonder dat er toxische effecten optraden. Daarom moet EROD inductie beschouwd worden als een biomarker voor blootstelling aan dioxineachtige contaminanten, en niet als een voorspeller van toxiciteit.

Ten derde wezen waarnemingen in de bursa van Fabricius erop dat contaminanten die momenteel (nog) in het milieu aanwezig zijn gevolgen kunnen hebben voor het immuunsysteem. Deze biomarker bleek erg gevoelig te zijn, en reageerde op contaminanten bij concentraties die lager waren dan die in het veld. Deze resultaten geven aan dat er meer onderzoek moet worden gedaan naar de rol van de verstoring van het immuunsysteem bij embryotoxiciteit in vogels.

ABBREVIATIONS

α-KGDH	α-ketoglutarate dehydrogenase
Ah receptor	arylhydrocarbon receptor
AHH	arylhydrocarbon hydroxylase
ANOVA	analysis of variance
ATP	adenosine triphosphate
Beq	bird egg equivalent
CYP450	cytochrome P450, hemoprotein with the ability to bind with carbon monoxide in the reduced form, and with a characteristic absorption at 450 nm
DD	developmental day
DDD	1,1-dichlorodiphenyldichloroethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene
DDT	1,1-dichlorodiphenyltrichloroethane
EMS	early mortality syndrome
EROD	ethoxyresorufin-*O*-deethylase
G6PDH	glucose-6-phosphate dehydrogenase
HCB	hexachlorobenzene
HpCN	heptachloronaphtalene
HxCN	hexachloronaphtalene
LD50	dose which is lethal to 50% of the population
M74	milieu 1974
NADP	nicotine adenine dinucleotide phosphate
NADPH	reduced form of nicotine adenine dinucleotide phosphate
OC pesticides	organochlorine pesticides
PAH	polyhalogenated hydrocarbon
PBDE	polybrominated diphenylether
PCB	polychlorinated biphenyl
PCB77	3,3',4,4'-tetrachlorobiphenyl
PCB126	3,3',4,4',5-pentachlorobiphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDE	polychlorinated diphenylether
PCDF	polychlorinated dibenzofuran
PCN	polychlorinated napthalene
PHAH	polyhalogenated aromatic hydrocarbon
POP	persistent organic pollutant
POP-REP	EU-project on effects of lipophilic persistent organic pollutants (POPs) on the reproduction of egg laying organisms
POV (dutch)	persistente organische verontreiniging
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TPP	thiamine pyrophosphate

CURRICULUM VITAE

Daphne Françoise de Roode was born in Rotterdam, the Netherlands, on October 26, 1974. In 1993, she graduated from the 'Openbare Scholengemeenschap de Ring van Putten' in Spijkenisse. In September of the same year she started her study Biology at the University of Utrecht (UU), with specializations in environmental chemistry, environmental toxicology and aquatic ecology. During this study she worked at the research institute of toxicology (RITOX, now: IRAS, institute for risk assessment sciences). Under supervision of dr. D.T.H.M. Sijm, she studied bioconcentration kinetics in green algae, and under supervision of dr. P. Mayer she conducted research on species differences in sensitivity towards the nonspecific action of toxic chemicals. In 1997, she worked at the Institute for Fisheries and Marine Biology at the University of Bergen (UB), Norway, where she carried out research on the influence of thyroid hormones and light regime on metamorphosis in halibut (supervision: dr. K. Pittman (UB) and prof. dr. H.J.Th. Goos (UU)). In January 1998 she graduated cum laude for her M.Sc. degree in environmental biology. From February 1998 to December 2001 she was employed as a Ph.D. student at Wageningen University (WU), with a detachment at the Institute for Forestry and Nature Research (now: Alterra), under supervision of dr. A.T.C. Bosveld (Alterra). During her Ph.D. she performed research on the effects of lipophilic persistent organic pollutants on reproduction in egg laying organisms, in collaboration with the University of Stockholm, the University of Jyväskylä and the University of Bergen. This project was financed by an EC grant and by programs of the Dutch Ministry of Agriculture, Nature Management and Fisheries. She also worked on projects funded by RIKZ. During her Ph.D. she attended several courses of the Postdoctoral training in Toxicology. Since January 2002 she is employed as a study director at NOTOX Safety and Environmental Research B.V. in 's-Hertogenbosch, the Netherlands.

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DANKWOORD

'I have seen that in any great undertaking it is not enough for a man to depend simply upon himself'.(Lone Man (Isna la-wica), late 19th century, Teton Sioux).

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