Effect of Organochlorine Pesticides on Maturation of Starfish and Mouse Oocytes

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Methoxychlor, lindane, and dieldrin are organochlorine pesticides that have been described as altering different reproductive functions in mammals and in invertebrates. However, few data have been published concerning the effects these pesticides have on oocyte maturation and fertilization. The aim of this study was to determine whether these compounds could affect maturation of mouse and starfish oocytes. We observed that germinal vesicle breakdown (GVBD) in starfish oocytes was significantly inhibited by the pesticides. Furthermore, formation of the first meiotic spindle and extrusion of the first polar body were also altered in mouse as well as in starfish. Our results suggest that the three pesticides act on common intracellular targets in invertebrates as well as in vertebrates.

Key Words: pesticide; methoxychlor; dieldrin; lindane; starfish; mouse; maturation; fertilization; meiosis.

Several reports published over the last three decades suggest that environmental exposure to synthetic estrogenic chemicals and related endocrine-active compounds might be responsible for a global decrease in sperm counts and decreased male reproductive capacity, and for breast cancer in women (Safe, 2000). Organochlorine pesticides have such estrogenic properties (Olea et al., 1998). These compounds have received the most attention because of their persistence in the environment, their ability to concentrate up the food web, and their continuous detection in the food supply and drinking water (Snedeker, 2001). They also seem to accumulate in organisms and then can cause endocrine disruption at environmentally realistic exposure levels (Vos et al., 2000). This report is focused on the effect induced by three organochlorine pesticides, methoxychlor (MXC), lindane and dieldrin, on maturation and fertilization of mouse and starfish oocytes.

These three compounds have been described as acting at different steps of reproduction in male as well as in female, from gonad functions and gamete production to embryo formation and pregnancy. Data have been obtained in various species, from invertebrates to mammals. Lindane, the γ-isomer of hexachlorocyclohexane, can have both estrogenic and antiestrogenic effects in the rat (Cooper et al., 1989). It has been reported to affect embryonic development in the rabbit (Seiler et al., 1994), early development of zebrafish (Gorge and Nagel, 1990), bovine (Alm et al., 1998), and mouse (Alm et al., 1996) embryos. MXC has been widely used as a substitute for DDT since it was shown to have a low mammalian toxicity and to be quickly degraded. However, it was then shown that this pesticide could also alter various reproduction functions such as ovary structure and function (Cummings, 1997; Eroschenko et al., 1995, 1997). It can affect embryonic development of mouse (Alm et al., 1998) or of sea urchin (Bresch and Arendt, 1977; Green et al., 1997). It alters initiation and maintenance of pregnancy in mice (Swartz and Eroschenko, 1998). Dieldrin also displays estrogenic activity and can affect ovarian function (Arnold et al., 1996).

Although pesticide effects on sperm and male fertility have been known for a long time, even taken as responsible for the decrease in male fertility in the world, few data have been reported concerning the effects of these pesticides on oocytes themselves, on maturation and fertilization. Maturation of bovine oocytes seems to be affected by 100 μM lindane and 75 μM MXC (Alm et al., 1998), and in vitro maturation induced by progesterone of Xenopus laevis oocytes can be inhibited by 10 μM MXC (Pickford and Morris, 1999). How they act on fertilization seems to be controversial. The fertilization rate of sexually mature female rabbits fed orally with lindane three times per week for 12–15 weeks was not modified (Seiler et al., 1994), neither was that of bovine oocytes matured in vitro in the presence of 75 μM MXC (Alm et al., 1998). On the contrary, 10 μM dieldrin reduces the fertilization rate of Bufo arenarum oocytes (Fonovich et al., 1995) and 100 μM MXC inhibits the first mitotic division of fertilized sea urchin eggs (Green et al., 1997). The crucial step that occurs during maturation is formation of a meiotic spindle. This allows a correct alignment and then segregation of chromosomes after an asymmetric cellular division and formation of a polar body. Some pesticides, such as benzimidazole derivatives, have been de-
scribed to alter meiotic and mitotic spindle in mouse oocytes (Can and Albertini, 1997), a result that could be expected since these compounds are well known to be microtubule-disrupting agents (discussed in Can and Albertini, 1997). Less was known concerning the oocyte sensitivity to lindane, MXC, and dieldrin and how these compounds could affect maturation. We have compared two different species, mouse and starfish, since both could be in contact in their environment with such pollutants. Mammals such as mice can ingest polluted food or water. Running water toward ground waters can also carry pesticides, and indeed, lindane, MXC, and dieldrin have been found from time to time in contaminated water near agricultural sites. Marine organisms such as starfish can then likely encounter these compounds due to land runoff. As a matter of fact, the three pesticides have been detected in seawater and sediments in various world areas (Zhulidov et al., 2000) and can then be ingested by marine populations. For example, MXC was detected in eggs of Morelet’s crocodile from Belize (Wu et al., 2000), dieldrin in eggs of puffins (Ingebrigtsen et al., 1984) and lindane in various marine mammals (Vetter et al., 1996). Results presented here show that lindane, MXC, and dieldrin are capable of altering oocyte maturation in mouse and starfish by acting at least on the meiotic spindle formation. This suggests that these pesticides act on intracellular targets common in invertebrates as well as in vertebrates.

**MATERIALS AND METHODS**

**Collection, Culture, and Maturation Oocytes**

**Mouse oocytes.** All protocols have been described in Polanski et al. (1998). To obtain immature oocytes arrested at prophase I of meiosis, the ovaries were isolated from 6- to 12-week-old Swiss female mice and transferred to prewarmed (37°C) M2 medium (Whittingham, 1971) supplemented with 4 mg/ml BSA and 50 µg/ml dibutylri cyclic AMP (dbcAMP) that prevents immature oocytes from undergoing germinal vesicle breakdown (GVBD). The ovarian follicles were punctured to release the enclosed oocytes, and immature oocytes displaying a germinal vesicle (GV) were collected. Groups of oocytes used to examine the effects of pesticides were washed out of dbcAMP to induce maturation, and were cultured in M2 medium containing each pesticide under paraffin oil at 37°C.

Metaphase II-arrested oocytes were recovered from mice super ovulated by intraperitoneal injections of pregnant mare’s gonadotropin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet) 48 h apart. Ovulated oocytes were released from the ampullae of oviducts between 12.5 and 14.5 h post-hCG. Cumulus were incubated in M2 medium containing each pesticide under paraffin oil at 37°C. GVBD and formation of the first polar body were observed under a Leica light microscope.

**Starfish oocytes.** All protocols have previously been described in Picard et al. (1988). The starfishes Astrostichopus aspruncus were collected near Banyuls, France, during winter, and kept in running natural sea water (NSW) before use. Prophase-arrested oocytes were prepared free of follicle cells by washing them several times in artificial Ca++-free sea water, transferred to NSW, and maturation induced by addition of 1 µM 1-methyladenine. GVBD and formation of the first polar body were observed under an Olympus light microscope.

**Immunofluorescence.** Fixation and labeling of mouse oocytes were performed as described in Kubia et al. (1992). We used the rat anti-tyrosinated α-tubulin monoclonal antibody YL1/2 and a fluorescein-conjugated anti-rat second antibody (Miles). The chromatin was visualized using propidium iodide (Molecular Probes; 5 mg/ml). Samples were observed with a Leica TCS4D confocal microscope.

Fixation and labeling of starfish oocytes was performed as follows. Vitelline membrane was removed by a 20-min digestion in 0.05% pronase in NSW, followed by 3 rinses in 1% BSA in NSW. Oocytes were extracted in a microtubule-stabilizing buffer (20% glycerol, 10 mM EGTA-KOH, 0.1 mM MgCl2, 100 mM MES pH 6.7, 1% NP40) for 1 h, then fixed in methanol for 1 h at room temperature. Anti-β-tubulin antibody (Amersham N 357) and FITC-conjugated anti-mouse IgG antibody (Amersham F0257) were diluted in TBS-Tween (150 mM NaCl, 50 mM Tris pH 7.4, 0.05% Tween 20). Oocytes were observed with an Olympus Fluoview confocal microscope.

**RESULTS**

**Effect of Lindane, Dieldrin, and MXC on Maturation of Starfish Oocytes**

Starfish oocytes were placed in NSW containing increasing concentrations of each pesticide. Final concentrations in NSW of each pesticide varied from 10 to 100 µM. Oocytes were then stimulated by addition of 1 µM 1-methyladenine. GVBD was observed 60 to 90 min after hormone addition and formation of the first polar body 30 min later. The highest concentration tested was 100 µM.

In seven different batches of oocytes, MXC induced a slight decrease in the percentage of maturation, as evaluated by the occurrence of GVBD, which progressively increased with the concentration of the pesticide, with a maximum effect at 100 µM (Fig. 1A). At this maximal concentration, the percentage of eggs showing GVBD was slightly reduced when compared to the control experiment (Fig. 1B). Lindane induced a more significant effect in all batches of oocytes, since 20 to 40 µM of this pesticide led to 30–70% decrease, depending on the batch of oocytes, of the percentage GVBD observed in the control experiment (Figs. 1A and 1B). Results obtained with dieldrin showed more variability. In five experiments, 100 µM dieldrin induced a slight decrease in the percentage GVBD in comparison with the control experiment (Figs. 1A and 1C), while this percentage GVBD was either dramatically or slightly increased in two other batches of oocytes at this high concentration (Fig. 1D). In fact, GVBD was slightly increased at low concentrations of dieldrin (Fig. 1A) in three batches of eggs that also showed a decrease in GVBD at 100 µM dieldrin (Fig. 1C). It is then possible that sensitivity to this pesticide varied with the batch of oocytes, explaining these contradictory data. All results reported above were obtained at the time of GVBD of the control experiment. We did not notice any further increase in percentage of GVBD at later times, suggesting that these lower proportions obtained in treated oocytes were not due to a delay in maturation induced by the pesticides.

Oocytes were taken 20 min after GVBD for fixation and observation of the spindle of first meiotic division. No significant alteration of the spindle was detected in any observed oocytes treated with 100-µM dieldrin (Fig. 2B) or lindane (Fig.
In oocytes treated with 100 μM MXC, asters were very small and poorly thick, and spindle were then hardly seen in these conditions (Fig. 2C). However, formation of a meiotic spindle in oocytes matured in the presence of 100 μM dieldrin was significantly delayed, since these oocytes were still at pro-metaphase with large asters when control oocytes had formed a meiotic spindle (Fig. 2A).

Observation of the polar bodies formation was very difficult, because of the altered size and/or shapes of the various figures of polar bodies obtained when oocytes were matured in the presence of 100 μM dieldrin, lindane, or MXC. Eggs treated with lindane (Fig. 3C) or dieldrin (Fig. 3D) expelled polar bodies that were either longer or smaller than those observed in control oocytes (Fig. 3A) or with abnormal shape. Finally, polar bodies of MXC-treated oocytes were very difficult to see because of their very small size (Figs. 3B1 and 3B2). It was then difficult to know whether some of these pesticide-treated oocytes had formed a polar body or not.

All oocytes matured in the presence of 100 μM dieldrin,
MXC, or lindane could be fertilized, as seen by the elevation of the fertilization envelope (not shown). However, none of them developed into normal embryos and all embryos died within 8 h after fertilization (results not shown).

**Effect of Lindane, Dieldrin, and MXC on Maturation of Mouse Oocytes.**

We first tested whether immature oocytes, arrested at prophase I of meiosis, would undergo maturation in M2 medium containing increasing concentrations of dieldrin, MXC, or lindane. Table 1 indicates that in all conditions and even at high concentrations of pesticides, GVBD occurred normally with the same efficiency and without any significant delay (not shown) when compared to the control experiment.

We then studied whether progression in the first meiosis cell cycle was altered when oocytes were cultured in the presence of the pesticides. Oocytes were arrested 4 h (Fig. 4) or 7.5 h (Fig. 5) after GVBD and fixed for immunofluorescence observation of the meiotic spindle. Figure 4B shows that oocytes, matured in the presence of 100-μM lindane, displayed a longer spindle than that observed in the control experiment (Fig. 4A). On the contrary, oocytes matured in the presence of the same concentration of dieldrin (Fig. 4C) or MXC (Fig. 4D) contained a first meiotic spindle smaller than that observed in control oocytes with a ball appearance. These abnormal spindles seen in dieldrin- and MXC-treated oocytes recovered a normal size 7.5 h after GVBD (Figs. 5B and 5C, respectively) that was similar to that seen in control oocytes (Fig. 5A). However, the spindle observed in lindane-treated oocytes displayed very long asters (Fig. 5B), which were not seen in control oocytes.

The presence of all pesticides did not prevent the formation of the first polar body 8 h after GVBD (Fig. 6). However, this event occurred with a 2-h delay in comparison with the control experiment in the presence of 100-μM dieldrin or MXC (Fig. 6).

Normally, development of matured oocytes stops at metaphase II of meiosis, where they wait to be fertilized. We observed that, in the presence of 100 μM dieldrin, lindane, or MXC, 30 to 40% of the oocyte population (results not shown obtained from 2 different experiments) expelled a second polar body. This suggests that the pesticides are capable of activating the oocytes.

**DISCUSSION**

The results presented here suggest that three organochlorine pesticides, dieldrin, MXC, and lindane, can alter oocyte maturation in mammals and in marine invertebrates. The effects were observed at relatively high doses of pesticides. Although it is unlikely to find such concentrations in contaminated areas, several reports indicate that land and aquatic organisms are capable of accumulating these compounds. Acute pesticide poisoning may also occur, particularly in developing states of Asia, South America, or Africa where control is poor, and where various pesticides, yet banned in North America or in Europe, are still used. Workers, who may not always wear protective gloves and respirators, may be exposed during manufacturing, handling, or spraying these pesticides. Obsolete pesticides can also be stocked in large quantities in uncontrolled locations, thus posing a serious threat to the environ-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μM)</th>
<th>n</th>
<th>Mean ± SE</th>
</tr>
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<tr>
<td>Control</td>
<td>0</td>
<td>8</td>
<td>77.8 ± 3.8</td>
</tr>
<tr>
<td>Lindane</td>
<td>10</td>
<td>2</td>
<td>79.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4</td>
<td>74.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>8</td>
<td>74.2 ± 6.03</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>10</td>
<td>2</td>
<td>80.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3</td>
<td>71 ± 8</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>5</td>
<td>72.2 ± 1.5</td>
</tr>
<tr>
<td>MXC</td>
<td>10</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
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<td>2</td>
<td>70.5</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>3</td>
<td>67.5 ± 8</td>
</tr>
</tbody>
</table>

*Note.* Results are mean ± SE of n experiments. Seventy-five to 200 oocytes were counted in each experiment.
ment and health of people living in the vicinity of such stockpiles. People, handling pesticides or not, and land or aquatic animals living in these areas can then be in contact with very high amounts of these compounds.

Various data indicate that in a variety of aquatic organisms exposed to contaminated water, the bio-concentration factor for lindane can exceed 1000 on a wet-weight basis. For example, the bio-concentration factors for brine shrimp, mosquito larvae, and the brook silverside (Halodelestes sicculus sicculus), exposed to lindane applied to the sand of a test aquarium, were 95, 220–383, and 600–1613, depending on the food chains used (Matsumura et al., 1976). Much higher bio-concentration factors have also been calculated. Hartley and Johnston (1983) determined a concentration factor for the freshwater clam, Corbicula manilensis, of 2610 on a lipid basis.

These pesticides can also accumulate in mammals including humans. Lindane was found at a concentration of 0.35 μM in the blood of a pregnant woman in the Indian countryside (Saxena et al., 1981). The β-HCH concentrations in the serum of a worker exposed for several years in a lindane-producing factory reached 2 μM (Brassow et al., 1981; Tomczak et al., 1981). High concentrations of dieldrin attaining 1.4 μM could be found in the blood of people after poisoning (Siyali and Simson, 1973). Moreover, another accumulation factor could be applied if we take into account the fact that these pesticides could accumulate from blood into tissue and organs. For example, the average concentration of dieldrin in the fat of workers in a manufacturing facility was 247 times greater than the mean plasma concentration there (Hayes and Curley, 1968). Dieldrin (Olea et al., 1998) and MXC (Olea et al., 1998) have been detected in reproductive organs such as ovaries, follicle fluids, oviduct, and uterine tissue. Therefore, oocytes could be in contact with very high concentrations of these pesticides in cases of poisoning. Finally, all experiments reported here were performed by incubating oocytes during short periods of time. Whether such treatment mimics several days or even months of exposure to lower concentrations of pesticides could not be performed in our laboratory, but would more accurately reflect the exposure levels found in the environment.

We observed that lindane, MXC, and dieldrin did not inhibit GVBD in mouse oocytes, even when used at 100 μM. This is contradictory to results obtained by Alm et al. (1998) in another mammal, showing that lindane and MXC affected resumption of meiosis of bovine oocytes in a dose-dependent manner. On the contrary, this event was significantly altered in starfish oocytes. Sensitivity to these pesticides of pathways leading to initiation of meiosis after the prophase exit of meiosis I may differ in animals, between mammals or between vertebrates and invertebrates. A decrease in intracellular cAMP level is responsible for meiosis resumption in mammals (Schultz et al., 1983), necessary but insufficient to exit from prophase I in starfish oocytes (Meijer et al., 1989). Therefore, another hypothesis is that pesticides may have altered a signaling pathway that leads to GVBD and occurs as well in those involving the decrease in cAMP in starfish oocytes. We also observed variability in the results obtained in starfish with dieldrin. Although low dieldrin concentrations slightly potentiated maturation, a small but significant inhibition in maturation occurred with increasing doses of this pesticide. This
could be due to sensitivity to this pesticide varying with the batch of oocytes.

A crucial step in meiosis completion is the formation of a meiotic spindle that allows segregation of homologous chromosomes that just underwent meiotic recombination while maintaining cohesion between sister chromatids. The first meiotic spindle is not located in the center of the oocyte and leads to an asymmetric cellular division with the extrusion of a polar body. This event was reported to be affected by other pesticides, in human oocytes by trichlorfon (Yin et al., 1998) and in mouse oocytes by carbendazim (Can and Albertini, 1997). Although we did not observe any alteration of the formation of this meiotic spindle by dieldrin in starfish oocytes, the length of the spindle was reduced in mouse oocytes matured in the presence of this pesticide, presenting a wool-ball aspect. A similar effect was induced by MXC, not only in mouse but also in starfish, where asters were very small and the spindle hardly seen, in oocytes matured in the presence of this pesticide. We also observed that these pesticides altered the formation of the mitotic spindle during the first mitosis of the fertilized sea urchin egg (Pesando et al., in preparation). Dieldrin and MXC seem then to act on similar targets involved in the formation of the meiotic and the mitotic spindle, in vertebrates as well as in invertebrates. These pesticides could directly alter microtubule polymerization. Dieldrin and MXC could also act on MPF (mitosis promoting factor) that is known to regulate microtubule dynamic (Verde et al., 1992) and activity of proteins associated with the spindle (Andersen and Karsenti, 1997).

Since the abnormal spindles seen in dieldrin- and MXC-treated oocytes recovered a normal size in mouse, MPF activity in treated oocytes could only have been delayed. Formation of short spindles has been observed in oocytes of KE mice where maturation is longer and MPF stimulation slower than in control oocytes (Polanski et al., 1998). A similar decrease in spindle size has been observed in mouse oocytes treated with MBC (Can and Albertini, 1997), but to our knowledge, such specific effects induced by dieldrin or MXC have not yet been reported, even in other biological systems. Finally, we observed that lindane treated oocytes showed a meiotic spindle that was formed either with a delay in starfish or with a larger size in mouse. As for dieldrin and MXC, lindane could alter MPF activity or microtubule dynamics during maturation. Enhancement of in vitro brain tubulin assembly by lindane has indeed been reported (Albertini et al., 1988). Disruption of microtubule organization in sea urchin embryos has also been recently reported to be induced by the herbicide chlorpropham (Holy, 1998).

As one could expect, abnormal spindles induced by each pesticide led to the extrusion of abnormal polar bodies, with altered size and/or shape in starfish. Lindane-treated oocytes extruded polar bodies with various shapes or sizes and those matured in the presence of MXC formed very small polar bodies. In some oocytes, it was difficult to know whether polar bodies could not be seen because of a very small size or because they had not been formed at all. Different complex mechanisms are involved in getting a normal asymmetric first meiotic division (Maro et al., 1994). Spindle migration and cortical polarization depends on a MAP kinase (mitogen-activated protein kinase) pathway, while spindle elongation at anaphase seems to be independent of this pathway (Verlhac et al., 2000). Dieldrin, MXC and lindane may then affect one of these events. This could be possible, in line with recent data showing that heptachlor, another organochlorine pesticide, can alter MAP kinase activity in human lymphocytes (Chuang and Chuang, 1998).

In mouse oocytes matured in the presence of dieldrin or MXC, a delay in polar body extrusion was observed. This is also probably due to alteration of kinases involved in the formation of the meiotic spindle that was first observed as abnormal and then recovered a normal aspect.

We did not observe any alteration of fertilization of starfish oocytes that had been matured in the presence of dieldrin, lindane, or MXC, even at high concentrations. All eggs used in experiments reported in Figure 1 fertilized and elevated a normal fertilization membrane. We obtained similar results in another invertebrate, the sea urchin, whose eggs could be fertilized in the presence of each pesticide (Pesando et al., in preparation). However, we did not observe any increase in polyspermy in all batches of eggs used in this report, contrary to sea urchin eggs (Pesando et al., in preparation). Finally, a large proportion of mouse oocytes treated with dieldrin, lindane, or MXC expelled a second polar body. This suggests that mechanisms, including normally occurring MAP kinase and MPF activities involved in arresting the metaphase of the second meiotic division, were altered. This activation of oocytes could also be due to modifications of Ca, that normally occur at fertilization (Stricker, 1999). Dieldrin has been reported to decrease the fertilization rate in Bufo arenarum oocytes by modifying the hydrolysis of phosphatidylinositol bisphosphate, an event that mediates Ca release from intracellular store (Fonovich et al., 2000). Effects of lindane on Ca, or on polyphosphoinositide metabolism have also been reported in various other biological models including human sperm (Silvestroni et al., 1997).

All these results strongly suggest that dieldrin, lindane, or MXC can alter maturation of oocytes of different species, from invertebrate to vertebrates. This can be at the origin of alteration of further development, as seen in starfish (results not shown), sea urchin (Pesando et al., in preparation), bovine (Alm et al., 1998), or mouse (Swartz and Eroschenko, 1998). It is then clear that exposure of animals reaching sexual maturity to these organochlorine pesticides can severely affect their fertility and lineage.

It is peculiar that lindane, MXC, dieldrin, trichlorfon, carbendazim, and chlorpropham all affect microtubule structures such as the meiotic spindle. This leads to the hypothesis that these compounds share a common structure capable of acting on a similar target, which would help to define a structure-
activity relationship (SAR), for the pesticides we have tested in this report (McKinney et al., 2000; Richardt and Benigni, 2002). No obvious key structural feature emerges from the structure of these pesticides; however, except for carbendazim, they are all chlorinated (Fig. 7). The marine environment is well known for its production of various halogenated compounds. For example, the 2-(3′,5′-dibromo-2′-methoxyphenoxy)-3,5-dibromoanisole was isolated from a marine sponge and is similar in structure to a polybrominated diphenyl ether (PBDE) (Reddy et al., 2002). Various organobromines, which were detected in marine mammal blubber from a number of species including seals, beluga, dolphins, or whales in various locations worldwide, would also be naturally produced (Tittlemier et al., 2002; Vetter, 2001; Vetter et al., 1996). Since starfish could probably ingest these halogenated compounds, it seems unlikely that halogenation alone could pose a serious toxicity problem for starfish. The effect that diet-derived chlorinated metabolites have on starfish oocytes will need to be evaluated in order to better understand the key structural components that are ultimately associated with toxicity.

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