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Uncertainties Associated with Assessing the Risk of an Endocrine Active Substance in the Canadian Environment

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A number of biological responses and multigenerational effects, mediated through the disruption of endocrine systems, have been observed in biota exposed to relatively low concentrations of environmental contaminants. These types of responses need to be considered within a weight of evidence approach in our risk assessment and risk management frameworks. However, including endocrine responses in an environmental risk assessment introduces a number of uncertainties that must be considered. A risk assessment of nonylphenol and nonylphenol polyethoxylates (NP/NPE) is used as a case study to demonstrate the sources and magnitude of some of the uncertainties associated with using endocrine disruption as an assessment endpoint. Even with this relatively well studied group of substances, there are substantial knowledge gaps which contribute to the overall uncertainties, limiting the interpretation within the risk assessment. The uncertainty of extrapolating from in vitro or biochemical responses to higher levels of organization or across species is not well understood. The endocrine system is very complex and chemicals can interact or interfere with the normal function of endocrine systems in a number of ways (e.g., receptors, hormones) which may or may not result in an adverse responses in the whole organism. Using endocrine responses can lead to different conclusions than traditional endpoints due to a variety of factors, such as differences in relative potencies of chemicals for specific endpoints (e.g., receptor binding versus chronic toxicity). The uncertainties can also be considerably larger and the desirability of using endocrine endpoints should be carefully evaluated. Endocrine disruption is a mode of action and not a functional endpoint and this needs to be considered carefully in the problem formulation stage and the interpretation of the weight of evidence.

Keywords: **risk assessment, endocrine disrupting substances, nonylphenol, nonylphenol polyethoxylates**

Introduction

The disruption of endocrine function in biota can lead to a variety of negative effects on growth, development, reproduction, behaviour or

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immunocompetency (National Academy of Sciences 1999; Di Giulio and Tillitt 1999). There has been concern that exposure to a wide variety of contaminants in the environment could result in unacceptable risk to ecosystems through their interaction with endocrine systems. The objective of this paper is to present and discuss some of the difficulties and uncertainties encountered in a complex environmental assessment of a chemical suspected of having the potential to disrupt endocrine functions. To illustrate these points, the risk assessment of nonylphenol (NP) and nonylphenol polyethoxylates (NPEOs) is used as a specific example to demonstrate some of problems and issues. This group of chemicals was recently assessed as a priority substance under the *Canadian Environmental Protection Act*. As part of the initial screening/problem formulation it was recognized that NP and NPEOs and their degradation products have the potential to interact with the endocrine system, but the significance and level of concern was not known.

The discussion is limited to only municipal effluents and the potential effects on aquatic biota. A more detailed discussion and presentation of other sectors and environments is available in the NP/NPE PSL-2 Risk Assessment (Environment Canada and Health Canada 2001) and the supporting background documents (Servos et al. 2000). This paper therefore does not represent a complete risk assessment, but serves as the basis for discussion of the difficulties and uncertainties of completing a scientific assessment of the potential risk of chemicals that may cause adverse effects through disruption of endocrine function. The risk assessment approaches using traditional endpoints of acute/chronic toxicity or endocrine-related endpoints are compared and the uncertainties identified.

Nonylphenol Polyethoxylates and Their Degradation Products in Municipal Effluents

Nonylphenol polyethoxylates occur as a complex mixture in final effluents of municipal treatment systems (Fig. 1; Lee et al. 1999; Bennie 1999). The range of concentrations as well as the relative proportions are dependent on the sources and degree and type of treatment (Maguire 1999). The distribution of nonylphenol polyethoxylates and their degradation products in municipal effluents reported across Canada is presented in Fig. 2 (see Servos et al. 2000). Typically the long EO chain NPEOs will degrade rapidly in treatment systems. Untreated effluents typically have elevated NP3–17EO concentrations and relatively high levels of NP and NP1,2EO, but treatment significantly reduces the concentration of NP3–17EO and NP in final effluents. As higher-chain-length NPEOs move through the treatment system, they are degraded to lower-chain-length NPEOs and nonylphenol polyethoxycarboxylates (NPECs) and ultimately to NP, which can be further degraded or sorbed to particles or sludges. Although NP1EO and NP2EO are created during treatment, concentrations of these transformation products are generally reduced in the final effluent of well-treated wastewaters. The nature of the inputs and type and degree of treatment strongly influence the concentrations and relative pro-

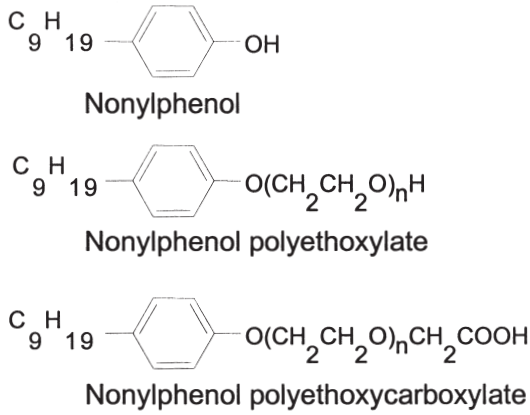

Fig. 1. The structure of nonylphenol poylethoxylates and their degradation products.

portions of NP/NPEO/NPECs released in final effluents. As the EO chain length decreases, a corresponding decrease in water solubility is observed. NP is, therefore, generally associated with organic particles and sludges in the treatment system. NPECs, however, are considerably more water soluble than the corresponding NPEOs and are present in the aqueous phase of final effluent. The concentration of NP1EC and NP2EC can therefore increase in concentration with increased degree of treatment, although extended treatment will also reduce the final concentration of NPECs in final effluents (Maguire 1999; Bennie 1999; Servos et al. 2000).

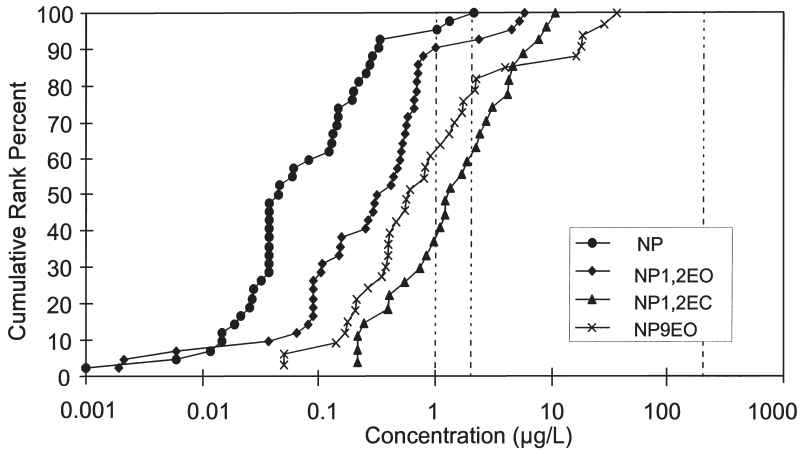

Fig. 2. Estimated environmental concentrations of nonylphenol poylethoxylates and their degradation products at municipal wastewater treatment sites (primary, secondary and tertiary). A dilution factor of 10 was applied to effluent concentrations as a conservative estimate of receiving water concentrations. The dotted line represents the levels of concern for NP, NP1,2EO and NP1,2,EC or NP9EO, respectively.

Effects of Nonylphenol Polyethoxylates and Their Degradation Products

Acute and Chronic Toxicity to Aquatic Biota

Several detailed reviews of the toxicity of alkyphenols (APs) and alkyphenol polyethoxylates (APEs) have been recently published by Talmage (1994), Staples et al. (1998) and Servos (1999). Although the data in the literature are scattered among many species, different test methods and chemicals, there is a consistent pattern in the toxicity. NP is relatively toxic (LC50, EC50) to fish (17–3000 µg/L), invertebrates (20–3000 µg/L) and algae (27–2500 µg/L) and chronic toxicity values (NOEC) as low as 6 µg/L in fish and 3.7 µg/L in invertebrates have been reported (see review by Servos 1999). Although there are considerably less data available for the NPEOs and NPECs there is an apparent increase in the toxicity of NP and NPEs with decreasing EO chain length. NPECs, which are much more water soluble, are much less toxic than corresponding NPEOs and have acute toxicity similar to NPEOs with 6–9 EO units. The relative toxicity of NP/NPEO/NPECs is summarized in Table 1 and is based on a comparison of acute and chronic toxicity data available in the literature, giving weight to data quality and consistency (Servos 1999; Servos et al. 2000).

Endocrine Disruption in Aquatic Biota

Numerous studies have demonstrated the ability of NPEOs and their degradation products to disrupt the normal function of the endocrine systems of various organisms (see Servos 1999 for a recent review). They have been reported to cause a number of estrogenic responses in a variety of aquatic organisms. NPEOs have been reported to have a variety of effects on fish, including altered growth of testes (Ashfield et al. 1998), altered steroid metabolism (Tremblay et al. 1998), intersex (Metcalfe and Gray 1997), histological changes (Miles-Richardson et al. 1999), and disruption of smoltificaton (Brown et al. 1998; Fairchild et al. 1999).

NP binds to the estrogen receptor resulting the expression of several responses both in vitro and in vivo, including the induction of vitellogenin in fish (Jobling et al. 1996). These effects occur at a range of concentrations similar to those at which chronic effects occur in fish and invertebrates (Fig. 3). The threshold of NP for induction of vitellogenin in rainbow trout was reported as 10 µg/L (Jobling et al. 1996), while the induction of mRNA for vitellogenin was reported at concentrations as low as 1 µg/L (Fent et al. 1999). Recent reports by Miles-Richardson et al. (1999) suggest that in fathead minnows, histological and biochemical effects can occur at concentrations approaching or below 1 µg/L. However, the significance of these responses is not fully understood, and the effects on the organism or population have not been determined. Recent work by Brown et al. (1998) has demonstrated that NP can affect smoltification, resulting in reduced growth and survival in Atlantic salmon (*Salmo salar*) after very short term (2 × 24-hour) exposures to concentrations as low as 20 µg/L (nominal). Intersex in Japanese medaka has been demonstrated at 50 µg/L (Gray and Metcalfe 1997).

Table 1. Summary of relative toxicity and relative estrogenicity based on endocrine disrupting effects

Chemical	Relative potency to E2[a] Vg induction, trout hepat. (Jobling and Sumpter 1993)	Relative potency to E2 YES assay (Burnison et al. 1998)	Relative potency to E2 YES assay (Routledge et al. 1996)	Relative binding to E2 receptor; Kd (M) (White et al. 1994)	Relative estrogenicity to NP estimate based on Jobling and Sumpter (1993)	Relative toxicity based on acute and chronic data (Servos et al. 2000)
NP	9.0 E-6	2 E-4	1.4 E-4	5 E-5	1	1
NP1EO					0.67	0.5
NP2EO	6.0E-6		6.6 E-6	0	0.67	0.5
NPnEO (9)	2 E-7		0		0	0.005
NP1EC	6.3E-6	8 E-6	4.0 E-5	2 E-4	0.63	0.005
NP2EC			4.0 E-5		0.63	0.005
OP	3.7 E-5	1.8 E-3	6.6 E-4	1.1 E-5	2.4	1

[a] E2 = 17β-estradiol.

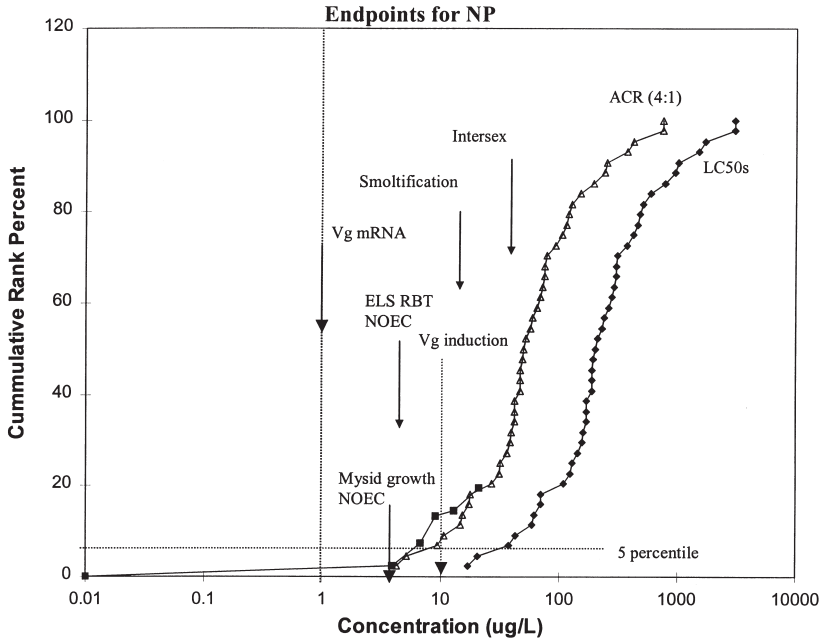

Fig. 3. The range in the responses of aquatic biota to NP reported in the literature. An acute to chronic ratio of 4:1 is typical of studies using the same species and test conditions reported in the literature (literature summarized in Servos et al. 2000).

Synthesis of vitellogenin is a biological response in fish that is mediated through binding of a chemical to the estrogen receptor. The threshold for this response is very similar to the LOEC for early life stage tests with rainbow trout, and only slightly below the thresholds for in vivo responses such as intersex and impaired smoltification. Although potential effects mediated through the estrogen receptor have been identified both in vitro and in vivo for NP in fish, this is only one mechanism by which a chemical such as NP can potentially interact with endocrine systems.

As with acute and chronic toxicity, there are few data available on the relative potency/estrogenicity of the other metabolites, and there is considerable discrepancy among the few existing studies. Jobling and Sumpter (1993) reported that using trout hepatocytes to measure induction of vitellogenin, NP2EO and NP1EC were 0.67 and 0.63 the potency of NP. These data of Jobling and Sumpter (1993), based on vitellogenin induction in trout hepatocytes, were used as the basis for determining the relative estrogenic potencies for comparisons in this exercise. Based on these data, both NP1,2EO and NP1,2EC are expected to be only slightly less estrogenic than NP. This contrasts with acute/chronic toxicity, where NP1,2EC is much less toxic than NP. However, considerable debate has emerged on the relative estrogenicity of these compounds. Other recent studies have reported considerably lower potency of NPEOs and NPECs in in vitro systems (Table 1). In transfected yeast cell assays (YES, with

hER), there is little or no binding of NP1,2EC to the estrogen receptor, suggesting a very low or zero potency (Burnison et al. 1998; Metcalfe et al. 2001). Mixtures of NP1,2EC did not cause ova-testes in Japanese medaka at concentrations similar to those that resulted in this response for NP (Metcalfe et al. 2001). Although this difference may be due to the characteristics of the in vitro assays, it does raise some uncertainty regarding the relative estrogenicity of these compounds. Biological responses resulting from exposure to these chemicals may also be mediated through mechanisms other than binding to the estrogen receptor. The discrepancies between potency estimates for NP1,2EC are particularly problematic and introduce considerable uncertainty into the assessment.

Risk Characterization at Municipal Effluent Sites

The concentrations of each of the nonylphenol polyethoxylate and the degradation products can be compared to the level of concern for each compound determined from the literature and applying an appropriate safety factor. The levels of concern selected based on chronic toxicity were 1 µg/L for NP, 2 µg/L for NP 1,2EO, and 200 µg/L for NP1,2EC and NP3-17EO. Less than 10% of the municipal effluent sites (dilution factor of 10:1 applied) have levels of individual NP or NPEs that exceed the level of concern based on acute/chronic toxicity endpoints.

Because NP and NPEs exist together as mixtures in effluents and environmental samples, the combined impact of the mixtures needs to be assessed. It was assumed that the lower-chain-length NPEs (NP1EO, NP2EO) and NPECs (NP1EC, NP2EC) have a mode of action similar to that of NP and that their effects are additive. The longer-chain-length NPEs (e.g., NP9EO) may differ from NP, because the mechanism of action is likely a physical surfactant effect so they were not included. A toxic equivalency approach was applied, which factored in contributions from NP as well as the lower-chain-length (1,2) NPEs and NPECs to determine the potential risk of the group as a whole. The toxicity of each metabolite relative to NP was determined from the available literature, and the TEQ was calculated as the sum of the exposure concentration (C_χ) of each compound multiplied by its relative potency (RP_χ). NP was used as a reference compound, because there were considerably more data available on the toxicity of this compound.

$$Total\ TEQ = C_\chi\ RP_\chi$$

The addition of NP1,2EO to the toxicity of NP increased the estimated total toxicity but only slightly increased the percentage of sites for which their was potential concern (Fig. 4). Despite the elevated levels of NP1,2EC in many effluents, adding them to the total made almost no difference to the total toxicity because of their much lower toxicity relative to NP.

A similar approach can be taken to estimate the total estrogenic potency. Based on a consideration of all of the available data, a level of concern of 1 µg/L NP was established, which corresponds to the threshold for induction of mRNA for Vg and by coincidence is the same value

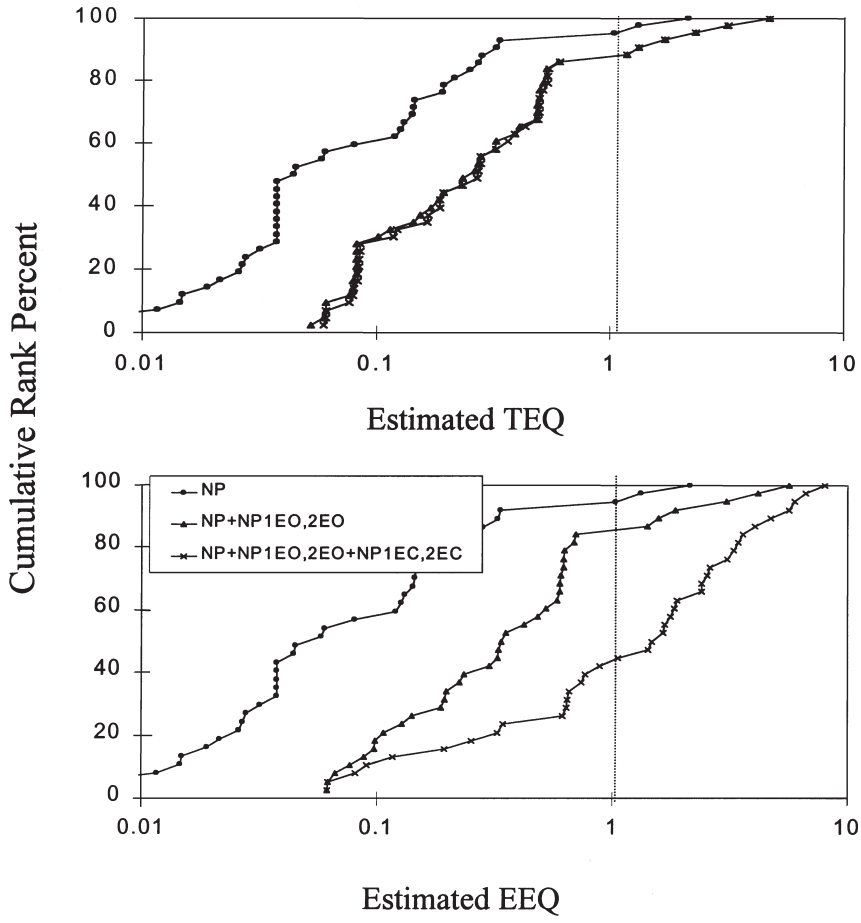

Fig. 4. Comparison of the environmental distribution (effluent concentrations/10) of nonylphenol poylethoxylates and their degradation products at municipal effluent treatment plant sites across Canada. Estimated total toxicity equivalency (TEQ) and estrogenic equivalency (EEQ) in µg/L for combined concentrations of NP, NPEOs and NPECs.

established for concern based on chronic toxicity of NP. When considered alone, concentrations of NP would not exceed the threshold for estrogenic responses, except in environments receiving primary treated municipal effluents (Fig. 4; note that an effluent dilution factor of 10:1 was applied). If the potential estrogenic effects of the NPEOs are added to the effect of NP, using a similar relative potency approach as above, then about 15% of the sites are expected to exceed the threshold of 1 µg/L. When the NPECs are also added, almost 60% of the municipal sites exceed the value of 1 µg/L. Many municipal effluents would be expected to cause vitellogenin induction, but they are not expected to exceed the threshold (10 µg NP/L for rainbow trout) in receiving waters after a 10:1 dilution, even when considered as a group. The large difference in the relative

potency of NPECs for acute/chronic toxicity, together with the elevated levels of NPECs in final effluents, considerably shifts the level of concern.

Considerations for Applying Endocrine Responses in a Risk Assessment

The differences in the NP/NPE risk assessment using acute/chronic toxicity and estrogenic responses was largely due to the difference between the relative toxicity and estrogenicity of NP1,2EC relative to NP. There is considerable uncertainty associated with the estimation of relative estrogenicity, and this value is critical to the conclusions. If the relative estrogenic potency of NP1,2EC is much less than that reported by Jobling and Sumpter (1993), as is indicated by some of the other recent studies, there would be very few Canadian municipal effluent sites, in the data set used, where the threshold for estrogenic-mediated responses would be exceeded.

The endpoints of concern should not be based on simply a response of the endocrine systems, but on a functional endpoint that is of concern based on the weight of evidence. The definition of an adverse effect should be link to a whole organism response rather than simply a response of a specific mode of action. Determining what the level of concern is can be difficult, as it can include protection of the individual, population or ecosystem and may involve public acceptability. If an animal can reproduce and develop normally but has some minor intersex or induction of mRNA for vitellogenin, is there an adverse effect or a concern? If the scope or capability of the organism to respond to other stresses or chemicals is compromised despite no detectable effects on the whole organism, is there a concern? These are important questions that should be debated and addressed early in the problem formulation stage of the risk assessment.

In this case study there were very few data available for comparing the relative estrogenicities of the compounds. It is not known how much uncertainty is associated with the extrapolation across different levels of organization. Simple receptor binding does not necessarily lead to biological effect of concern in vivo. Studies are also often carried out in a minimal number of test species. How much uncertainty is associated with extrapolating data across species? An interesting example would be related to predicting the effects of estrogenic compounds on smoltification of Atlantic salmon, based on standardized testing in rainbow trout. Perhaps, with sufficient knowledge of mechanisms, these responses would have been recognized, but it is unlikely that this depth of understanding will be available in the near future. Recognizing that there is considerable uncertainty in many aspects of the scientific assessment, quantifying the uncertainty in the risk assessment will be a difficult challenge.

A substance such as NP/NPEs is actually a very complex mixture of chemicals. The relative composition of the chemicals may differ drastically in different effluents or environments. The widely different physical and chemical properties result in large differences in their treatability, behaviour and fate in the environment. In the case of NPEOs, one compound may be the precursor to the others leading to very complex inter-

actions and unexpected behaviour, such as increasing concentrations of selected compounds with increasing degrees of treatment. The differences in chemical and physical properties can lead to different exposures for biota to each compound, and each may have different toxicity (including differences in responses such as estrogenicity). In the current assessment, only the water route was considered, but it is likely that NP is sorbed to particles, therefore having very different exposure pathway and bioavailability relative to NPECs which are relatively water soluble.

Substances such as NPEOs seldom occur alone in the environment. Related compounds such as octylphenol and octylphenol polyethoxylates almost always occur with them as a complex mixture. In addition unrelated compounds are often found in complex mixtures that have similar mechanisms of action or toxicity. In municipal effluents other compounds such as natural and synthetic estrogens have been identified, which also bind to the estrogen receptor and cause similar biological responses, e.g., intersex (Desbrow et al. 1999; Routledge et al. 1999). How to account for these compounds that may also add to the potential effects in effluents is a difficult task. The endocrine systems are very complex and binding to the estrogen receptor (or estrogenicity) is only one mechanism of many. How these chemicals interact with other receptors or other compounds with similar mechanism or other modes of action to cause responses in the whole organism remains a major knowledge gap.

Even for a substance with a relatively large amount of data, there is considerable uncertainty in making a scientific assessment based on an endocrine mechanism. It is questionable whether focusing on a single mechanism (receptor binding) or mode of action (endocrine disruption) is an adequate approach. The endocrine issue has made us recognize the need to pay particular attention to the subtle effects that environmental contaminants may have on growth, development, reproduction, behaviour, or immuno-competence that might not have been recognized in the past. Unfortunately there is typically very little information available for subtle or multigenerational effects, even for well-studied chemicals and substances. Focus on a single mechanism or mode of action should not distract the assessor from addressing the overall risk to the environment and the weight of evidence should continue to be used to make scientifically defensible decisions. However, information on specific mechanisms may be useful in guiding the assessors to ask appropriate additional questions and acquire additional data. Reducing the uncertainty associated with extrapolating from endocrine responses measured in vitro or in vivo to functional endpoints of reproduction and development is necessary to improve the reliability of future scientific assessments of the risk of priority substances in the environment.

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