Comments of the
European Council for Alkylphenols and Derivatives
and the
Alkylphenols & Ethoxylates Research Council
On the REACH Annex XV Report: Proposal for Identification of a Substance as a CMR Cat. 1A or 1B, PBT or vPvB, or a Substance of an Equivalent Level of Concern: 4-Nonylphenol, branched and linear

Submitted October 17, 2012

Executive Summary

The European Council for Alkylphenols and Derivatives (CEPAD) and the Alkylphenols & Ethoxylates Research Council (APERC) jointly submit these comments in objection to the Annex XV Report on 4-nonylphenol(4-NP), which proposes classification of this compound as a Substance of Very High Concern (SVHC) under Regulation (EC) 1907/2006 (REACH).

The criteria for SVHC are listed under Article 57as:

> (a) substances meeting the criteria for classification as carcinogenic category 1 or 2 in accordance with Directive 67/548/EEC;
> (b) substances meeting the criteria for classification as mutagenic category 1 or 2 in accordance with Directive 67/548/EEC;
> (c) substances meeting the criteria for classification as toxic for reproduction category 1 or 2 in accordance with Directive 67/548/EEC;
> (d) substances which are persistent, bioaccumulative and toxic in accordance with the criteria set out in Annex XIII of REACH;
> (e) substances which are very persistent and very bioaccumulative in accordance with the criteria set out in Annex XIII of this Regulation; and
> f) substances — such as those having endocrine disrupting properties or those having persistent, bioaccumulative and toxic properties or very persistent and very bioaccumulative properties, which do not fulfill the criteria of points (d) or (e) — for which there is scientific evidence of probable serious effects to human health or the environment which give rise to an equivalent level of concern to those of other substances listed in points (a) to (e) and which are identified on a case-by-case basis in accordance with the procedure set out in Article." (emphasis added)

The Annex XV Report for 4-NP proposes these compounds as SVHC "because they are substances with endocrine disrupting properties for which there is scientific evidence of probable serious effects to the environment which give rise to an equivalent level of concern to those of

other substances listed in points (a) to (e) of Article 57 of REACH." The proposal is based primarily on the following arguments cited in the summary of the Report (pg. 5):

1.) "*There is "strong evidence from high quality studies of endocrine mediated adverse effects in fish species*"; and
2.) "*Results for amphibians provide indications that effects in other taxa may be endocrine mediated i.e. caused by an estrogen-like mode of action, too.*"

These comments will address why these two arguments, and others provided in the Annex XV Report for 4-NP, are scientifically inadequate to justify identifying this compound as SVHC.

In addition to scientific concerns, CEPAD and APERC have process concerns with the fact that there are currently 54 substances nominated for identification as SVHC under public consultation with the same deadline for comment on October 18, 2012. Managing such a large number of dossiers within a short time frame will certainly be a challenge for both the ECHA Secretariat and the Member State Committee, which raises questions about the how thoroughly public comments will be considered.

Also, CEPAD and APERC object to the proposal to nominate 4-NP as SVHC because it raises a fundamental policy issues that require further clarification under REACH before proceeding with SVHC nominations under Article 57(f). There are currently no criteria for what constitutes "probable serious effects to the environment" from endocrine modes of action, or any other mode of action, established under REACH. In fact, the EU Commission still has ongoing activities related to the development of a definition of "endocrine disruptors" and criteria for their assessment under REACH. Therefore, it is premature to consider nominations for compounds as SVHC on this basis in advance of these policy developments at the level of the European Commission.

4-NP does not meet the criteria for SVHC under Article 57(a) through (e). 4-NP does not meet the criteria for PBT or vPvB as determined by several governmental authorities. (Joint Meeting of the Competent Authorities for the Implementation of Council Directive 67/548/EEC, 2001; Joint Research Centre, Institute for Health and Consumer Protection, TC NES Subgroup on identification of PBT and vPvB Substances). 4-NP also does not meet the criteria for probable serious effects for SVHCs as defined under CMR Cat. 1 or 2; it also does not meet criteria for the weakest category, CMR Cat. 4. (ECB, 2002) However, 4-NP is toxic to aquatic organisms. As discussed later in these comments, the estrogenic mode of action is not the sole mode of action for 4-NP, and the most sensitive aquatic ecotoxicity end points for 4-NP are not definitively linked to an estrogenic mode of action. Furthermore, considering that the adverse effects of 4-NP on aquatic organisms is already controlled through existing regulatory instruments such as the Water Framework Directive, which has an Environmental Quality Standard (EQS) for 4-NP, and the Integrated Pollution Prevention and Control (IPPC) Directive, there does not appear to be a need to further prioritize a nomination for this compound as SVHC on the basis of concern for the toxicity to the environment

In addition, there is a long-standing EU Market and Use Directive (M&U Directive) for Nonylphenol and its Ethoxylates first issued under DIRECTIVE 2003/53/EC OF THE

EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 June 2003 amending for the 26th time Council Directive 76/769/EEC and now incorporated into the REACH regulation as (EC) No 1907/2006, Annex XVIII. The M&U Directive for 4-NP/NPE was based on concern for the aquatic toxicity of 4-NP and restricted the marketing and use of 4-NP and its ethoxylates in the EU for major wide dispersive uses that result in a direct discharge to the aquatic environment.

In the absence of EU criteria, the Annex XV Report for 4-NP proposes that this compound meets the threshold for "equivalent level of concern" under Article 57(f); however it does not provide an adequate scientific basis to support a case that this compound rises to a level of concern that is equivalent to a CMR Category 1, 2, PBT or vPvB compound. Article 57(f) of REACH states "substances giving rise to an equivalent level of concern" should have "scientific evidence of probable serious effects to human health or the environment"; therefore it seems that the intention of the regulation is that SVHC should be reserved for substances for which there is an adequate scientific basis to support a finding of effects that are both probable and serious.

As will be discussed further in these comments, 4-NP has only weak estrogenic activity that is 1,000 to 1,000,000 fold less potent than the potent estrogens17ß-estradiol (E2) and 17α-ethynylestradiol (EE2), when calculated based on numerous reliable *in vitro* and *in vivo* studies. Adverse apical effects caused by 4-NP are not "clearly endocrine mediated", but rather are indicative of general toxicity (baseline narcosis) possibly coupled with very weak estrogenic activity. The effects of 4-NP are clearly not comparable to other more potent steroidal estrogens. Results of aquatic toxicity tests conducted on 4-NP and potent estrogens show that effects on acute lethality, reproduction, growth, and development are very different for 4-NP and EE2.

The following comments elaborate on these points and respond to the specific arguments provided in the Annex XV Report for 4-NP to demonstrate that while 4-NP may have some weak estrogenic activity, it does not demonstrate adverse environmental effects that give rise to an equivalent level of concern as CMR, PBT or vPvB compounds.

1.0 The IPCS Definitions distinguish between "endocrine disruptor" and "potential endocrine disruptor"; and the OECD Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption provides testing guidance, not policy guidance for regulatory prioritization or action related to endocrine active compounds.

As noted above, CEPAD and APERC object to the proposal to nominate 4-NP as SVHC because it raises a fundamental policy issues that require further clarification under REACH before proceeding with SVHC nominations under Article 57(f). There are currently no criteria for what constitutes "probable serious effects to the environment" from endocrine modes of action, or any other mode of action, established under REACH. In fact, the EU Commission still has ongoing activities related to the development of a definition of "endocrine disruptors" and criteria for their assessment under REACH. These activities include discussion about an endocrine disruptor hazard classification scheme and consideration of the potency, adversity of effects and requirement to review the weight-of-evidence for endocrine disruptors.(Kortenkamp, 2011).

In the absence of EU definitions and guidance on the "probable serious effects to the environment" from endocrine modes of action, the Annex XV Report for 4-NP relies on the IPCS definition and the OECD Guidance Document Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption. (OECD, 2011) However, the Report overlooks the following features of these sources on endocrine disruption.

1.1 The IPCS Global Assessment of Endocrine Disrupting Compounds distinguishes between an "endocrine disruptor" and a "potential endocrine disruptor".

The IPCS in their Global Assessment of Endocrine Disrupting Compounds (EDCs) stated that:

> *"An **endocrine disruptor** is an exogenous substance or mixture that alters function(s) of the endocrine system and **consequently causes adverse health effects in an intact organism,** or its progeny, or (sub)populations; and*
>
> *A **potential endocrine disruptor** is an exogenous substance or mixture that possesses properties that **might be expected to lead to endocrine disruption** in an intact organism, or its progeny, or (sub)populations."* [emphasis added] *(*WHO, IPCS,2002).

Most noteworthy, for a chemical to be considered an endocrine disruptor, the endocrine mediated effects must occur in a whole organism and they must be adverse. IPCS stresses that: *"Endocrine disruption is not considered a toxicological end point per se but a functional change that may lead to adverse effects."* Thus, chemicals that show some endocrine modulation (e.g., estrogenic, androgenic, thyroidogenic) are not necessarily endocrine disruptors and should <u>not</u> be considered to be such.

The U.S. Environmental Protection Agency makes this point in their two-tiered Endocrine Disruption Screening Program (EDSP), where results from Tier 1 screening tests (which may indicate potential endocrine activity) are not indications of definitive "endocrine disruption". Rather results from Tier 2 multigenerational toxicity tests with intact organisms determine whether a substance may cause endocrine-mediated effects through or involving various hormone systems. (US EPA, EDSP, 2012)

It should be noted that the WHO-IPCS report recognizes that not all endocrine active compounds should be considered as endocrine disruptors. Certainly, the potency, efficacy, as well as exposure concentrations, will affect the probability that a compound will actually cause serious endocrine disruption. In addition, the influence of other co-occurring modes of action will determine whether endocrine activity or disruption is the primary mode of action. The Annex XV Report for 4-NP overlooks these aspects of the IPCS Global Assessment of Endocrine Disrupting Compounds.

1.2 The OECD Guidance Document Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption is intended as a tool to assist regulatory authorities to interpret assays and explicitly states that it is not intended to prejudge or constrain regulatory actions in its testing guidance.

The objectives and scope of the OECD Guidance Document were defined such that the document would be a tool to support regulatory authorities by helping to interpret assay results and suggesting possible additional studies for reducing uncertainty. The Guidance Document specifically states that "the guidance should not prejudge or constrain what regulatory actions may be taken by a member country and should not suggest a testing strategy". (OECD, 2011). The Guidance Document also notes "the use of many of these tests for determination of toxicity due to endocrine disruption (hazard and risk assessment) for mammals and wildlife is rather new, and therefore the guidance given is considered to be subject to changes based on new evidence". The Guidance Document is intended to be a "living" document that will be updated as the science in this area evolves and should not be relied upon as a regulatory standard or criteria.

In the OECD Guidance Document, an endocrine disrupter (ED) has been defined according to WHO IPCS, 2003 as described above, and "it is acknowledged that many other definitions exist (*e.g.* Weybridge Conference, 1996) but the WHO (2003) definition has been used as a working definition for this document because it covers both human health and wildlife populations". The Guidance Document points out that this definition is widely used but not universally accepted. The OECD Guidance Document "operationally defined the term 'possible ED' to mean "a chemical that is able to alter the functioning of the endocrine system but for which information about possible adverse consequences of that alteration in an intact organism is uncertain".

This raises the need for the development of EU criteria for determining probable and serious effects in aquatic species, regardless of mode of action. Endocrine mediated reproductive effects that are "known" or "presumed" based on supporting data that show they occur in the absence of other interfering toxicological mechanisms may rise to a similar level concern as Cat. 1 or 2 (based on DSD classification criteria) reproductive toxicant, particularly when effects occur at exposures that are environmentally relevant. However, reproductive effects that occur at only concentrations that are so high that other modes of toxicity are also occurring or that are not environmentally relevant should not be considered to be of equivalent concern to CMR Cat. 1, 2 or PBT or vPvB compounds.

2.0 4-NP is very weakly estrogenic, operates by multiple modes of action, and does not result in serious adverse endocrine-mediated effects comparable to the potent natural estrogen, 17β-estradiol (E2) and synthetic (EE2) estrogens.

2.1 4-NP is considerably less potent than natural (E2) and synthetic (EE2) estrogens based on in vitro estrogen activity screening tests and endpoints from in vivo tests.

Valuable information about the relative potency of endocrine active compounds relative to the known potent estrogens E2 and EE2 are provided by various in *vitro* screening tests and *in vivo* tests with intact organisms. Endpoints from *in vivo* tests included gonadal histology, kidney lesions, vitellogenin, sex ratio, hatching success, and swim-up. Relative potencies from these studies can be approximated by concentrations of 4-NP and E2 or EE2 used as a positive control that gave comparable results. Overall, the data show that 4-NP is approximately 10^3–10^6-fold less potent than the endogenous estrogen E2 or the synthetic estrogen EE2, depending

on the species and endpoint investigated (Jobling and Sumpter 1993; Lee and Lee 1996; Islinger *et al*. 1999; Metcalfe *et al*. 2001; Dussault *et al*. 2005; Balch and Metcalfe 2006; Lin and Janz 2006; Zha *et al*. 2007, 2008).

2.2 Examination of the estrogenic potency and aquatic toxicity of natural estrogen (E2) and synthetic estrogen (EE2) provides an understanding of what constitutes a highly potent estrogen**.**

The Annex XV Report on 4-NP emphasizes the estrogenic activity of 4-NP as the basis for "equivalent concern" in its assessment of the compound's aquatic toxicity. In light of this concern, it is of use to first examine effects on aquatic organisms from known endocrine active substances for which the mode of action is indisputable – natural estrogen (E2) and a synthetic and therapeutic estrogen (EE2). Relevant studies are summarized in (Table 2) and more fully presented with experimental details in the attached Table A.

Table 2. Summary of Aquatic Toxicity Studies for 17α-ethynylestradiol (EE2) and 17β-estradiol (E2)

Study Type	NOEC Apical Endpoints	NOEC Secondary Endpoints	Comments	References
Screening or Short Term Reproduction Fathead minnow Sheepshead minnow Zebrafish	Survival, reproduction, fertilization spawning success, hatch success, sex ratio: ≤0.005 to 0.20 μg/L	VTG, GSI, kidney lesions, gonadal histopathology, testis-ova: 0.0002 to 0.010 μg/L	Spawning, fertilization, mating behavior most affected. Effects on gonadal histopathology severe	Miles-Richardson 1999 Zilloux 2001 Van den Belt 2001 Coe 2010
Life Cycle Studies Fathead minnow Zebrafish	F0 fertilized eggs to adult exposure, survival, growth, reproduction: 0.0001 to 0.016 μg/L F1 sex ratio: 0.0002 μg/L	Secondary sex characteristics, testis-ova, gonadal histopathology, VTG: 0.001 to 0.004 μg/L	Complete feminization of adult fish. No male external characteristics or gonadal tissue.	Lange 2001
	F0 fertilized eggs to adult exposure, survival, growth, reproduction: 0.0003 to 0.010 μg/L F1 fertilization: 0.0003 μg/L	Not reported	Ratio of acute LC50 to fertilization NOEC: 1700 μg/L/0.0003 μg/L = 5.73 million.	Wenzel 2001
Whole Lake Studies Fathead minnow Pearl dace	Whole lake exposed for 3 years to 0.005 μg/L EE2 Complete collapse of fathead minnow populations. Decreased abundance of pearl dace populations.	VTG Edema in ovaries Inhibition of male gonad development, intersex tissues, kidney lesions	Complete feminization of males, inability to reproduce.	Kidd 2007 Palace 2006

E2 and EE2 have been found to be of generally similar potencies to each other. EE2/E2 ratios range from 0.19 to 1.9 depending on the screen used (Kidd et al., 2007). E2 and EE2 are especially potent when compared to the weak estrogenic activity of 4-NP.

The natural and synthetic estrogens E2 and EE2, respectively, cause significant and severe effects on freshwater and saltwater fish. As described in more detail below, a signature set of aquatic effects from E2 and EE2 may include: complete feminization of male gonads; induced failure to differentiate into males; reduced reproduction capabilities; severe histological effects on gonadal tissues; and even collapse of whole populations in a lake continuously dosed with EE2 for three years.

A series of short term reproduction assays showed that survival, reproduction, fertilization, spawning and hatch success, and sex ratio can be significantly altered at estrogen concentrations down to 0.005 µg/L. Secondary endpoints such as gonadal weights and histopathology, testis-ova, and kidney lesions were affected at even lower concentrations, down to 0.0002 µg/L.

Life cycle studies with fathead minnow and zebrafish that included exposure to EE2 over the full life cycle showed similar effects at 0.001 to 0.016 µg /L and 0.0003 to 0.010 µg /L, respectively. Second generation NOEC were seen at concentrations as low as 0.0002 to 0.0003 µg/L (Lange R., 2001; Wenzel et al., 2001). Secondary endpoints including secondary sex characteristics, testis-ova, gonadal histopathology, and VTG had NOEC in the 0.001 to 0.004 µg/L range. In both studies, and presumably due to the strong estrogenic potencies of these compounds essentially complete feminization of males and/or male characteristics were reported.

In order to differentiate the relationship of endpoint effects caused by a narcotic mode of action and those mediated by an estrogenic mode of action, Wenzel et al . (2001) examined the ratio of the acute LC50 of EE2 (1,700 µg /L) and the most significant reproduction NOEC (fertilization success, NOEC 0.0003 µg /L). The acute-to-chronic ratio of the two is 5.73 million. This very high ratio is indicative of the mode of action associated with the synthetic estrogen EE2 and very different from the ratios that are calculated with 4-NP (22 to 116) as is discussed below.

In a whole lake study, Kidd (2007) and colleagues dosed a whole lake (0.005 to 0.006 µg/L) with EE2 for 3 years. Fathead minnow populations became completely feminized, reproduction halted, and the population collapsed. The abundance of pearl dace fish was also decreased. These two species of fish are predominant in the lake. As noted in laboratory studies, severe effects on gonadal tissues were found. Male gonadal tissue development was stunted or halted, edema was noted in ovarian tissues and lesions were found in kidney tissue.

In summary, the effects on aquatic organisms by potent estrogenic substances such as E2 and EE2 collectively add up to a predictable, cohesive signature of effects, which are consistent with the known mechanism of action for these compounds. Natural development of male gonadal tissue is halted, differentiation into male characteristics is blocked, male secondary sex characteristics are prevented from developing, and gonadal tissues are severely compromised

and damaged, reproduction within natural populations can be halted, and effects that are mediated by estrogenic activity occur at concentrations that are millions of times lower than those where narcotic effects occur. As a scientifically rigorous review of relevant studies shows (see Section 2.3 below), the effects of 4-NP on aquatic organisms are very different from the effects from potent estrogens.

2.3 4-NP affects acute lethality and chronic reproduction, growth and development endpoints very differently than natural (E2) or synthetic (EE2) estrogens.

The Annex XV Report for 4-NP attempts to link effects reported for 4-NP from all the studies with fish to an endocrine disruption mode of action. Comparison of studies examining 4-NP and natural (E2) and synthetic (EE2) estrogens demonstrates that there are great differences between the effects that they cause to aquatic organisms.

Estrogenic activity within intact aquatic organisms due to exposure to the natural and synthetic estrogens E2 and EE2 occurs at concentrations that are orders of magnitude below the threshold for systemic toxicity. These exposures result in a suite of responses that are collectively linked to the estrogenic mode of action. In a life cycle study with EE2 and zebrafish, Wenzel et al. (2001) reported the 96-h LC50 (narcosis-based toxicity) for EE2 to be 1700 µg/L and the NOEC for fertilization success to be 0.0003 µg/L. The authors calculated the ratio of the two values to be 5.73×10^6. It is clear for EE2 that the reproduction, growth and development endpoints are affected by a different mechanism of action (endocrine) than the acute lethality (narcosis) endpoints. Given the potency of natural and synthetic estrogens, this is expected, and hence the very high ratio is understandable.

4-NP does not affect the same endpoints in aquatic organisms in the same way that natural and synthetic estrogens act. Data from three studies with 4-NP demonstrate that the reproduction, growth and development endpoints are mainly indicative of narcosis-type mode of action, possibly coupled with a very weakly estrogenic mode of action.

Using rainbow trout, Brooke et al. (1993a) reported a 96-h LC50 of 221 µg/L. Ackermann et al. (2002) reported a no observed effect concentration (NOEC) from a one year exposure to embryonic, larval, and juvenile rainbow trout of 10.17 µg/L based on hatching success and sex ratio. The ratio of the lethal effect concentration (221 µg/L) to the reproductive NOEC of 10.17 µg/L is 22. Using fathead minnows, Brooke et al. (1993a) reported a 96-h LC50 of 128 µg/L. Giesy et al. (2000) reported a NOEC of 1.6 µg/L based on egg production with fathead minnows. The ratio of the acute lethal concentration of 128 µg/L and the NOEC of 1.6 µg/L for the reproductive endpoint is 80. Using Japanese medaka, Kashiwada et al. (2002) reported a 96-h LC50 of 950 µg/L. Yokota et al. (2001) conducted a 1.5 generation test with Japanese medaka reporting a lowest NOEC for F1 sex ratio of 8.2 µg/L. The ratio of the acute lethal effect concentration of 950 µg/L and the NOEC of 8.2 µg/L is 116. Thus, the ratio of acute lethality LC50 values and the NOEC of the lowest apical NOEC for reproduction, growth or development endpoints are fairly consistent across the species rainbow trout, fathead minnows, and Japanese medaka. Across the three species, the ratios range from 22 to 116, which are approximately 50,000- to 260,000-fold lower than the ratio of 5.73 million calculated for EE2.

Together these data show that 4-NP has a very different mode of action than the natural and synthetic estrogens with effects on survival resulting from narcosis being at least as critical to the organisms exposed to 4-NP as other modes of toxicity. While biomarkers such as VTG indicate that estrogen receptor binding is occurring while exposed to 4-NP, the endpoints related to growth, development, and reproduction are only a factor of 22 to 116 from short term acute toxicity and in the same range as caused by longer term lethality. Hence it is possible that effects on growth, development, and reproduction for 4-NP are more indicative of narcosis-type mode of action, possibly coupled with a very weakly estrogenic mode of action. Taken together, the acute and chronic datasets for 4-NP and EE2 are very different. This conclusion is supported by the assertion by Schwaiger et al. (2000) based on laboratory exposures of Common carp (Cyprinus carpio) to 4-NP, "…4-NP-induced general toxic effects might outbalance the relatively weak estrogenic effects of this compound…" as ecologically relevant endpoints such as reproduction may be "…also due to toxic effects leading to an impairment of the general health condition of the fish."

2.4 4-NP operates under multiple modes of action, not just a weak estrogenic mode of action.

While 4-NP has weak estrogenic activity that is 1000 to 1,000,000 fold less potent than 17ß-estradiol (E2) and 17α-ethynylestradiol (EE2) (Coady et al., 2010), adverse apical effects caused by 4-NP are not "clearly endocrine mediated". The assumption that effects on biologically complex, apical endpoints, such as reproduction, are mediated by a single mode of action, in this case one that is endocrine mediated via the estrogen receptor, is not reasonable for alkylphenols. In estrogenic mixture studies with estrogens and alkylphenols, the phenomenon of decreased fish reproduction due to alkylphenol exposure alone was clearly not solely attributed to estrogen-like activity (Brian et al., 2007). Investigations using gene array technologies to specifically compare 4-NP and estradiol (E2) gene transcription profiles have established that 4-NP has additional modes of action that are independent of the estrogen receptor (Larkin et al., 2002; Ruggeri et al., 2008; Watanabe et al., 2004). Molecular evidence in both mammalian and fish models have demonstrated that the alkylphenols influence a greater suite of genes than estrogens (Ruggeri et al., 2008; Watanabe et al., 2004). For example, 425 genes were differentially expressed in liver tissue from zebrafish exposed to 10^{-7}M 4-NP, while 153 genes were differentially expressed in liver tissue from zebrafish exposed to 10^{-7}M E2 (Ruggeri et al., 2008). Of the 30 most differentiated genes affected by 4-NP compared to controls, only 1/3 of these genes were also altered among E2-exposed fish, and then not all in the same direction of change (Ruggeri et al., 2008). In mice, nonylphenol activated more genes than E2 in liver tissue, and the activated genes in the livers of 4-NP-exposed mice were distinct from estrogen-responsive genes (Watanabe et al., 2004). These molecular studies of gene activation illustrate that 4-NP has multiple modes of action, of which weak estrogenic activity is one, and the apical effects noted on aquatic organisms, such as decreased growth, survival and reproduction, are not necessarily directly or solely attributed to 4-NP's weak estrogen receptor binding activity.

3.0 The concern expressed in the Annex XV Report on 4-NP is for reproductive or developmental effects in aquatic organisms caused by an endocrine mechanism; however it does not provide adequate rationale for the leap between findings of

weak 4-NP estrogenic activity and reproductive effects seen in some fish studies.

The endpoints that are considered to indicate an estrogen agonist mode of action are summarized on page 49 (Table 18) of the Report. Several of these endpoints are more apical in nature and can be observed naturally and also under various stressful conditions for fish. Oocyte atresia normally occurs in fish as a part of the natural reproductive cycle, so an understanding of the normal background level of this phenomenon is important. Oocyte atresia and testicular degeneration also occur in fish when exposed not only to an estrogenic compound, but also in response to compounds with known androgenic or anti-androgenic modes of action (USEPA, 2007). Therefore it appears that oocyte atresia and testicular degeneration, in and of themselves, are not indicative of a particular endocrine mode of action (*i.e*. estrogen receptor agonism). It is also established that stressful situations resulting in sustained increases in a fish's cortisol levels can depress sex steroid concentrations, which in turn can cause delayed gonadal development, lowered VTG levels, and depressed secondary sex characteristics in fish (Milla et al., 2009; Aluru and Vijayan, 2009). Therefore, these endpoints are not necessarily specific to an endocrine mediated mode of action, but will respond when fish are stressed, whatever the cause may be.

In various studies with medaka fish, increased VTG was observed in males at concentrations of 4-NP $\geq$ 5.4 µg/L, altered histopathology of the gonads (specifically occurrence of testis-ova) generally occurred at concentrations of 4-NP $\geq$ 8.2 µg/L. The occurrence of testis-ova in the gonads was more marked when fish were exposed during the time period of sexual differentiation, which occurs before hatch. Incidences of ova-testis were less frequent and occurred at higher concentrations when exposure to 4-NP was initiated post-hatch. Sex ratios in medaka fish were altered toward a greater proportion of females at concentrations of 4-NP $\geq$ 17.7 µg/L. These results indicate that effects noted in medaka may be due to the weak estrogen binding activity of 4-NP. However, the apical effects that have a relevance to medaka populations (*i.e*. altered sex ratio), occur at concentrations well above the current Environmental Quality Standard (EQS) of 0.3 µg/L for 4-NP in surface waters under the Water Framework Directive as well as above the Predicted No Effect Concentration (PNEC) of 0.6 µg/L that is listed in the REACH Dossier for 4-NP and was based on a species sensitivity distribution model (ECHA, 2012).

In studies with fathead minnows of various ages, increased VTG was observed at concentrations of 4-NP $\geq$ 8.1 µg/L, altered histopathology of the gonads and altered secondary sex characteristics generally occurred at concentrations of 4-NP $\geq$ 1.6 µg/L. Apical endpoints, such as reproduction and survival, were affected among fathead minnows, including early life stages, at concentrations of 4-NP $\geq$ 14 µg/L. While behavioral effects of 4-NP exposure on male fathead minnows were described by Schoenfuss et al., 2008, namely nest holding behavior, the magnitude of the noted effects among 4-NP-exposed fish was low (*i.e.* 5-10% of 4-NP-exposed fish were outcompeted by control males) and arguably not of biological relevance. The biological relevance of such a small effect is questionable, especially for an endpoint as variable as nest holding behavior, which has not been validated in a standardized fish testing guideline. Also notable in the Schoenfuss et al., 2008 study is the fact that patchy and high mortality rates were observed among tested fish at concentrations of 4-NP that are lower than those known to result in fish mortality

(*i.e.* 58% survival in 0.15 µg/L 4-NP treatment group), thus questioning the husbandry practices for the fish in this study. Again, the apical effects that have a relevance to fathead minnow populations (*i.e.* reproduction and survival), occur at concentrations well above the current environmental quality standard of 0.3 µg/L for 4-NP in surface waters under the Water Framework Directive.

A quote from the section on fathead minnow results in the Annex XV Report for 4-NP (page 64) states, "Although apical effects started at similar or even lower concentrations compared to biomarker responses, it seems very likely that they are estrogen mediated." There really is no support for this statement, since 4-NP has multiple modes of action apart from weak estrogen receptor binding, as has been demonstrated by several gene array and other molecular based mode of action studies with 4-NP (see section 2.4). There is no definitive link with the weak estrogenic effects noted among fathead minnows (*i.e.* effects on VTG and reproductive tissues) and the effects on apical endpoints (*i.e.* reproduction and survival) which occur at overlapping concentrations. The most sensitive apical effect for fathead minnows that was statistically different from controls in a validated study was based on mortality in an ELS study (Ward and Boeri, 1991).[1] There is no clear link in this study with fathead minnow mortality and an estrogenic mode of action.

A total of four 4-NP exposure studies with zebrafish were examined in the report, however, 3 of the 4 studies were deemed a Klimisch score 3, which indicates that these studies are "not reliable" (Klimisch, 1997). However, the endpoints from these studies are still used in a weight of evidence evaluation of the effects of 4-NP on zebrafish. With inappropriate controls included in the test design and high control mortality rates, the study endpoints are compromised and should not be included in a weight of evidence approach for determining the effects of 4-NP on zebrafish. From the one study determined acceptable for consideration (Lin and Janz, 2006), VTG was increased among male and female fish at 100 µg/L 4-NP, testicular tissue was skewed to earlier stages at 10 µg/L 4-NP, and the apical effects of 1) no detectable males based on gonadal histology and 2) decreased swim up success were noted at 100 µg/L 4-NP. Altered sex ratios compared to controls were also observed at 10 µg/L 4-NP (58% females at 10 µg/L 4-NP vs 30% females in controls), however since the sex ratio was skewed in the control group, the meaningfulness of this observation is questionable. The apical effects that have a relevance to zebrafish populations (*i.e.* altered sex ratio and survival), occur at concentrations well above the current environmental quality standard of 0.3 µg/L for 4-NP in surface waters under the Water Framework Directive.

Various studies with rainbow trout exposed to 4-NP were summarized, and effects on increased VTG levels were noted at concentrations as low at 1.05 µg/L. Effects on gonadal tissues were noted at concentrations of 4-NP $\geq$ 30 µg/L, and effects on apical endpoints including growth and survival, were noted at concentrations of 4-NP $\geq$ 10.3 µg/L. Additionally, the effect of reduced sperm volume was noted in one study (Lahnsteiner et al., 2005) starting at concentrations of 4-NP $\geq$ 0.13 µg/L; however, reproduction was not assessed in this study, so it is unknown if this decrease in sperm volume affected reproduction levels in

[1] The Ward and Boeri 1991 study with fathead minnows is scored in the Annex XV Report for 4-NP as Klimisch score 4 (insufficient information). The Klimisch score for this study should be1. A copy of the unpublished study is provided as Attachment B to these comments for verification.

rainbow trout. In addition, this endpoint is not well-studied, therefore the usefulness of the endpoint for predicting effects for population health is not known. The lowest apical endpoint noted in among the toxicity studies with rainbow trout was the reduced growth of rainbow trout at 10.3 µg/L in an early life stage test (Brooke, 1993).[2] This apical endpoint is not clearly related to an estrogenic mode of action. Fish growth can be affected by various modes of action and reductions or delays in growth are not necessarily estrogen-mediated. In fact, the OECD Guidance Document on the Assessment of Chemicals for Endocrine Disruption states, "… an effect solely on growth or survival, while potentially of concern from the viewpoint of environmental risk assessment, would not lead to a conclusion that the chemical is an ED in fish." (OECD, 2011). Again, the apical effects that have a relevance to rainbow trout populations (*i.e*. growth and survival), occur at concentrations well above the current environmental quality standard of 0.3 µg/L for 4-NP in surface waters under the Water Framework Directive.

In studies with guppies and mosquito fish, 4-NP exposure resulted in similar findings with increased VTG at concentrations of 4-NP ≥ 10 µg/L, changes in gonadal tissues at concentrations ≥ 0.5 µg/L, and effects on apical endpoints at concentrations of 4-NP ≥ 5 µg/L. While skewed sex ratios were noted at concentrations of 4-NP ≥ 50 µg/L, significant effects on growth were noted at lower concentrations of 5 µg/L. As stated previously, decreased growth is not specific to an estrogenic mode of action. As seen with the other fish species, apical effects that have a relevance to viviparious fish populations (*i.e*. growth), occur at concentrations well above the current environmental quality standard of 0.3 µg/L for 4-NP in surface waters under the Water Framework Directive.

Taken together, the fish studies do indicate that 4-NP has a mode of action that is consistent with a relatively weak binding affinity to the estrogen receptor when compared to the endogenous ligand, E2. Increased levels of VTG, alterations in gonadal histopathology, and altered sex ratios in fish are consistent with this mode of action. However, the most sensitive apical endpoints among fish toxicity studies with 4-NP are based on decreased growth and survival, particularly of early life stage fishes. These endpoints are not clearly linked to an endocrine mode of action, and are not estrogen agonist specific. There are other modes of action associated with 4-NP as is evidenced by the suite of genes that are differentially activated in gene array and other molecular based investigations of the mode of action of 4-NP.

4.0 The Annex XV Report for 4-NP does not provide adequate scientific support for its statement that amphibian data provided in the Report support a conclusion that 4-NP causes endocrine mediated effects in amphibians and other taxa.

The Annex XV Report for 4-NP states "Results for amphibians provide indications that effects in other taxa may be endocrine mediated i.e. caused by an estrogen-like mode of action, too."

[2] The Brooke, 1993 rainbow trout study is scored as aKlimisch score of 4 in the Annex XV Report for 4-NP due to inaccessibility of the report. This is a US EPA study that should be scored as Klimisch 1. A copy of the report is provided as Attachment C to these comments.

However, most of the amphibian toxicity studies summarized in the Annex XV Report for 4-NP were not designed to specifically assess endocrine activity of 4-NP. Measured endpoints in the amphibian toxicity studies commonly included tadpole growth (length and weight measurements), metamorphic progress (an apical endpoint, sensitive to multiple types of stressors), survival, and incidence of developmental abnormalities. None of these endpoints are specifically indicative of estrogen receptor agonism. The report indicates that pigmentation (melanocyte differentiation) can be estrogen responsive. However, changes in pigmentation among both amphibians and fish can occur for multiple reasons, including as a response to stress or altered temperature (Green and Baker, 1991; Fernandez and Bagnara, 1991); therefore changes in pigmentation should not be interpreted as estrogen agonist specific. Taken together, the concentrations of 4-NP resulting in effects to amphibian populations are similar to the concentrations that cause effects to fish populations. Apical endpoints in amphibians (such as growth, malformations, and survival) were altered at concentrations of 4-NP $\geq$ 100 µg/L, which is much higher than the current EQS of 0.3 for 4-NP under the Water Framework Directive. More specific endpoints related to estrogen receptor agonsim (i.e. gonad histology and sex ratio) were measured in an toxicity study with 4-NP using wood frogs (*Rana* sylvatica) and northern leopard frogs (*Rana pipiens*) (Mackenzie et al., 2003). However, there are multiple issues with this study that indicate the data should be used with care, and these are: 1) In the study design, wood frog exposures were not replicated due to limited availability of the eggs, 2) only two concentrations of 4-NP were evaluated in the studies, limiting the interpretation of the data as a full dose-response evaluation for 4-NP was not possible with the data set, 3) measured concentrations of 4- NP indicate that the static renewal test design system was not adequate to maintain exposure concentrations (only 9% of nominal 4-NP concentrations were measured, on average), 4) mortality in the leopard frog study was high across all treatment groups including controls (ranging from 40 to 58%), and 5) the authors themselves point out several uncertainties with the intersex and sex ratio endpoints observed in the study indicating the occurrence of intersex among controls and the unanticipated sex ratio responses of some of the chemical exposures. Therefore, this study should not be viewed as providing definitive data for the effects of 4-NP on amphibians in regard to estrogen specific modes of action. Additional information for 4-NP in amphibians can be gleaned from a more recent, well-conducted, chronic study with a close 4-NP, analogue 4-tert-octylphenol (OP). In a study with OP and *Xenopus tropicalis* tadpoles, animals were continuously exposed to 0, 1.1, 3.3, 11, and 36 µg/L OP from Nieuwkoop and Faber stage 46 to adulthood (a total of 31 weeks) with a sampling time-point at the completion of metamorphosis as well (Porter et al., 2011). In this study, no significant deviation from the control sex ratio was observed for either sampling period, suggesting minimal to no effect of OP exposure on gonad differentiation. No effects in the adult frogs were observed for mortality, body mass and size, liver somatic index, estradiol and testosterone serum levels, sperm counts, or oocyte counts. The development and growth of oviducts, was observed in males exposed to OP, and VTG levels were increased among juvenile male frogs (sampled at the time of metamorphic completion), but not in adult male frogs at the close of the exposure at 31 weeks. The transient increase in male vitellogenin and the presence of oviducts in some male frogs exposed to OP indicates weak estrogenic activity of OP, however Porter et al (2011) concludes

that the highest OP concentration used, 36 µg/L, was at or below the no observed effect level (NOEL) for toxicity in *X. tropicalis*.

In the Annex XV Report for 4-NP, several studies were included in the weight of evidence evaluation of 4-NP effects on amphibians, even though these studies are not reliable for use. In particular, the study by Kloas et al. (1999) is considered not valid as the authors failed to control temperature during the test, had no analytical confirmation of test concentrations, had skewed sex ratio in controls, and used improper statistics. The study with the same species by Van Wyk et al. (2003) is considered not relevant as the authors employed intraperitoneal injection of 4-NP as the means of dosing, which is not a relevant route of exposure for species and test material. Therefore, these studies should not be included in the weight of evidence evaluation for 4-NP, not should they be used to justify estrogenic effects of 4-NP in other taxa.

5.0 The scientific evidence presented in the Annex XV Report for 4-NP is in some cases based on invalid studies and flawed study interpretation and the overall assessment does not adequately support a case that this compound rises to a level of concern that is equivalent to a CMR Cat. 1A, 1B, PBT or vPvB compound.

The bulk of the Annex XV Report is devoted to presenting a case that 4-NP is a substance of "equivalent concern"; however the scientific analysis used to make that case is circumstantial, simplistic, incomplete, and in some cases wrong. Specific examples of these problems are given in the discussion of the individual studies below. A full analysis of the available aquatic toxicity data for this compound does not give rise to a conclusion that serious aquatic effects are probable or would be comparable to a Cat. 1 or 2 CMR, PBT or vPvB substance.

In Chapter 5 of the Annex XV Report for 4-NP, the aquatic hazard assessment is presented. The assessment introduces a number of studies mostly with fish, amphibians, and invertebrates and identifies potential findings that might be indicative of endocrine activity. Prior to analysis of any given study, the authors of the report gave each study a study quality score according to the principals of Klimisch (1997). The scoring of the various studies by the authors of the report was inappropriate in many cases. Types of errors include scoring studies as valid (scores 1 or 2) that used no statistics, lacked replication, or had poor control performance. Additionally, several studies were scored as "not valid" (3), but the studies were used anyway in the Report. Studies properly scored as "not valid" (3) cannot be used in a hazard assessment.

Some studies were inappropriately scored as "use with care" and should have been scored as "3" or "not valid". Ashfield et al. (1998) conducted a 400+ day study with female rainbow trout measuring length and weight, plus ovarian weight. Test concentrations were never measured over the course of the test, so the authors had no way of knowing test concentrations, if concentrations were stable or drifted well beyond nominal, and did not even measure stock solution concentrations to verify that test chemical was delivered to the test vessels. In addition, length and weight measurements varied significantly during the 431 day test. At some time

points, lengths and weights were higher than controls, at other times they were lower. There was no consistent dose related pattern of changes and most changes were within 10% of control. The dosing is suspect and the growth effects were small and not consistent. The study should properly be scored as "3", not valid.

Lahnsteiner et al. (2005) exposed adult rainbow trout, and subsequently eggs, to 4-NP in the reported range of 0.13 to 0.75 µg/L. Concentrations in test vessels and stock solutions were never measured. The authors had no confirmation at all of the concentrations to which the fish were exposed. Concentrations were only estimated. Effects on semen volume and fertilized egg survival were reported at all concentrations. These data are dramatically different from all other data with rainbow trout. For instance, Ackermann et al. (2002b) reported no effects on gonad maturity stage, hatching success, mortality, or growth at 10.17 µg/L. Brooke (1993) reported effects on growth at 10.3 µg/L, effects on survival at 23.1 µg/L, and no effects on time to hatch or survival of hatched embryos at 23.1 µg/L. Jobling et al. (1996) reported effects on testis growth at 54.3, but not 20.3 µg/L. The results of Lahnsteiner et al. (2005) should be scored as "3" and considered not valid because the authors had no idea what concentrations were in the test vessels and their results are not supported by other studies cited in the Annex XV Report.

The study by Ward et al. (2006) examined social behavior of juvenile rainbow trout exposed to 4-NP at 40 and 80 µg/L. Some effects on social behavior were reported at both concentrations. However, the study employed no replication. It is not possible to demonstrate statistical significance without replicates and thus, the study by Ward et al. (2006) should be scored as "3", not valid. Schoenfuss et al. (2008) conducted competitive spawning assays using fathead minnows of either 8 months of age (Exp. 1) or 9 months of age (Exp.2). The authors reported significant effects on competition at concentrations as low as 0.15 µg/L in Exp. 1 using 8 month old fish, but only at 11 µg/L and higher in Exp. 2. The extraordinary difference in results between the two nearly identical experiments that used adult fathead minnow of either 8 or 9 months of age suggests significant experimental flaws or that such competitive spawning assays are too highly subjective to be useful for hazard or risk assessment. Due to the great differences in results from two nearly identical experiments, the study should properly be scored as "3", not valid.

Arslan and colleagues (Arslan and Parlak 2007; Arslan et al. 2007) reported the results of studies with two species of sea urchin, *Paracentrotus lividus* and *Arbacia ixula*.Spermiotoxicity, genotoxicity, and embryo toxicity were assessed in both species of sea urchins exposed to seven treatments of 4-NP ranging from 0.937 to 18.74 μg/l (nominal concentrations, 4-NP was not measured). In the spermiotoxicity test, sea urchin sperm were exposed to 4-NP delivered in dimethylsulfoxide for 30 minutes, then mixed with viable eggs. Fertilization success was measured and the embryos were assessed for malformities and qualitatively scored in terms of development stages. The genotoxicity test was conducted by assessing embryos for mitotic abnormalities after 5 hours of exposure to 4-NP at the concentrations listed above. In the embryo toxicity test, sperm and eggs were added to the treatments together and exposed to 4-NP treatments throughout 72 hours of embryonic development. Spermiotoxicity effects on larval development of *P. lividus* were observed at all concentrations of4-NP down to 0.937 μg/l (Arslan *et al*. 2007). These results are in contrast to other work reporting effects on the sea urchin, *P. lividus,* in which sea urchin sperm was exposed to 4-NP for 72 hours after which an

EC50 of 270 μg/l based on sperm toxicity was recorded (Ghirardini *et al*. 2001). In light of conflicting data, there is uncertainty associated with the results with sea urchins reported by Arslan *et al*. (2007). Embryotoxic effects among both species of sea urchins were observed at all concentrations of 4-NP down to 0.937 μg/l (Arslan *et al*. 2007; Arslan and Parlak 2007). However, due to potential solvent effects and a lack of test chemical measurements in the static tests (in which test chemical concentrations are likely to be less consistent than in flow-through tests), the use of the Arslan *et al.* (2007) and Arslan and Parlak (2007) studies for hazard or risk assessment should be considered with caution.

6.0 As stated under Article 57(f) scientific evidence of probable serious effects to human health or the environment is necessary to classify a compound as a SVHC; the very low estrogenic potency, multiple modes of action, and low environmental occurrence and concentrations do not support a conclusion of 4-NP of <u>probable and serious</u> environmental effects from this compound.

Categorization as SVHC should be reserved for substances for which the weight-of-evidence supports a finding of <u>probable and serious</u> effects. As discussed previously, 4-NP, at most, has weakly estrogenic activity and the available data do not support a conclusion that this compound is of equivalent concern to a CMR Cat. 1 or 2, PBT or vPvB compound. As discussed below, concentrations of 4-NP in European waters are significantly below NOEC and LOEC values for adverse apical effects found in studies on 4-NP in aquatic species. In addition, studies that examine estrogenically active substances in wastewater treatment plant effluent found that if detected, 4-NP and alkylphenols generally, contributed only minimally to the aggregate estrogenicity.

6.1 Concentrations of 4-NP in European surface waters do not support concern for <u>probable and serious</u> effects in aquatic species

4-NP Monitoring data were collected between 2007 and 2009 in the context of the water framework directive (DGEnv, 2009). In the water dissolved fraction mean concentrations of 0.040 µg/L (maximum 0.460 µg/L, median 0.030 µg/L) were observed. These dissolved concentrations are well below all NOEC and LOEC from the screening, short term reproductive, and life cycle studies with 4-NP that ranged from approximately 1 to 180 µg/L. These data provide further support to the evidence that 4-NP does not cause "probable serious effects" to the environment that are equivalent to CMR Cat. 1 and 2, PBT, or vPvB substances.

6.2 Contribution of 4-NP to Measured Estrogenicity of Effluents or Surface Waters

Numerous studies have examined the extent to which measurable estrogenic activity in wastewater treatment plant (WWTP) effluents is attributable to natural and synthetic compounds (e.g., E2 and EE2) or to other constituents (e.g., 4-NP or other alkylphenols).

Desbrow et al. (1998) used a sample fractionation system to isolate fractions of WWTP effluent samples that were tested for estrogenicity using a YES assay. Estrogenic fractions were found to contain natural and synthetic hormones including E2, estrone, and EE2. Alkylphenolic compounds were not detectable in the estrogenic fractions.

Most studies employed in vitro screening assays such as YES assays and concentration measurements from whole effluents to assess relative contributions to estrogenicity. Korner et al. (1999) and Aerni et al. (2004) reported that the contribution of 4-NP in German WWTP effluents was <3% and 2%, respectively, of the total estrogenicity. In WWTP effluents in the US, Drewes et al. (2005) reported that nearly all of the estrogenic activity was attributable to natural and synthetic hormones. Similar findings have been reported for WWTP effluents in Australia, New Zealand, and China, (Leusch et al., 2006; Jin et al., 2007; Lu et al., 2011).

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