

Appendix A

Comments to the BLM Draft PEIS for Vegetation Management Using Herbicides on 17 Western States.

Nonylphenol Ethoxylates and its Degradates

Need for Analysis of NPE

The lack of analysis and requirements for chronic and endocrine effects is possibly the greatest failing in the proposed specifications for water enhancers. This is especially true for NPE and its degradates.

The primary risk assessment document currently used by the FS for NPEs is entitled "USDA Forest Service. 2003. Human and Ecological Risk Assessment of nonylphenol polyethoxylate based (NPE) surfactants in Forest Service herbicide applications. Pacific Southwest Region." It has not been published in a scientific publication or been peer reviewed. It is sometimes cited in FS publications as "Bakke 2003", but its proper citation, and as it will be cited here, is as "USDA 2003". This document is poorly written, outdated, and incorporates unscientifically applied analysis for risk assessment purposes. It is thoroughly analyzed and critiqued below.

Updated Information

It should be noted that a very recent study from Nov 2004 (Bulayeva 2004, see below) has shown NPEs to be many times more toxic than found in the comprehensive "Risk Assessment Report. 4-Nonylphenol (Branched) and Nonylphenol", European Union (EU RA 2002). This highlights one of the most crucial points about assessing risk from NPEs and endocrine disruption. Because the science and understanding of endocrine disruption is relatively new, the body of scientific data concerning both NPEs and endocrine effects is growing exponentially. When the EU decided to ban the manufacture of NPEs, it did so by relying on data that showed toxic effects in the low parts per billion range. New studies show that NPEs are toxic in the parts per trillion range, producing significant negative effects with a onetime exposure. It is possible that in a few years, new data will lower these threshold levels even more.

Background

Nonylphenol ethoxylates (NPEs) are nonionic surfactants, identified numerically by their ethoxylate chain length, and are a class of a broader group of compounds known as alkylphenol ethoxylates (APEs). NP is a chemical intermediate composed of a phenol ring attached to a lipophilic straight or, more usually, branched nonyl group.

NPEs are high volume chemicals that have been used for more than 40 years as detergents, emulsifiers, wetting agents and dispersing agents, and as such are ubiquitous in the environment. Nonylphenol polyethoxylate containing products are used in many sectors, including textile processing, pulp and paper processing, paints, resins and protective coatings, oil and gas recovery, steel manufacturing, pest control products and power generation. A variety of cleaning products, degreasers and detergents are also available for institutional and domestic use. NPEs are also used in cosmetics and other consumer applications.

NPEs and their degradation products (e.g., nonylphenol [NP]) are not produced naturally. Their presence in the environment is solely a consequence of anthropogenic activity. The mechanism of degradation is complex, but, in general, there is an initial loss of ethoxylate (EO) groups from the original moiety. Under aerobic and anaerobic treatment

conditions, biodegradation to more persistent, toxic and hormonally active degradates occurs. These products include NP, nonylphenol ethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), nonylphenoxyacetic acid (NP1EC) and nonylphenoxyethoxyacetic acid (NP2EC) (Environment Canada, 2001).

NPE and its degradates have been shown to produce a wide range of toxic effects, both acute and chronic. Analysis of toxic effects will be divided into two sections, endocrine system effects, and non-endocrine effects.

Overview of Endocrine Effects

The endocrine system consists of a set of glands, the thyroid, parathyroids, testes, ovaries, adrenal, hypothalamus, pancreas, pineal, and pituitary glands, as well as other chemical regulators; and the hormones they produce, such as thyroxine, oestrogen, testosterone and adrenaline, which coordinate and regulate internal communication in cellular organisms. Endocrine cells release chemical messengers, known as hormones, which are carried into contact with target cells in the body. Interactions between the hormone and particular recognition features (receptors) in the cell, trigger pre-existing cellular responses that may result in effects on growth, behavior, development, or reproduction, as well as numerous other critical biological functions.

The currently recognized definition of endocrine disrupting chemicals is;

“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. 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” (WHO, 2002).

While EDs have been clearly associated with developmental, reproductive, neurobehavioral and other health problems in wildlife and laboratory animals, experts suggest these compounds may also affect humans in similar ways.

“Analysis of the human data by itself, while generating concerns, has so far failed to provide firm evidence of direct causal associations between low-level (i.e., levels measured in the general population) exposure to chemicals with EDCs and adverse health outcomes. It is difficult to compare and integrate results from diverse human studies, because data are often collected at different time periods, using different experimental designs and under different exposure conditions. Often exposure data are completely lacking. Of particular concern is the lack of exposure data during critical periods of development that influence later functioning in adult life. Furthermore, the concentrations and potencies of endogenous hormones and phytoestrogens are generally higher than those of exogenous chemicals. Despite these difficulties, exposure to EDCs has been suggested to play a role in adverse health outcomes, and concerns remain” (WHO 2002).

“Overall, the biological plausibility of possible damage to certain human functions (particularly reproductive and developing systems) from exposure to EDCs seems strong when viewed against the background of known influences of endogenous and exogenous hormones on many of these processes. Furthermore, the evidence of adverse outcomes in wildlife and laboratory animals exposed to EDCs substantiates human concerns. The changes in human health trends in some areas (for some outcomes) are also sufficient to warrant concern and make this area a high research priority, but non-EDC mechanisms also need to be explored” (WHO 2002).

Effects to wildlife are well documented and no longer an issue under dispute. Concerning effects to humans, most current reviews of EDs use cautionary statements, that data is as yet inadequate to reach a judgement. In sum, however, the weight of the evidence not only shows that a problem exists, it has far reaching implications. Human

impacts beyond isolated cases are already demonstrable. They involve impairments to reproduction, alterations in behavior, diminishment of intellectual capacity, and erosion in the ability to resist disease. But human epidemiology is biased toward false negatives in the search for health effects of endocrine disruption. It will be exceedingly difficult, if not impossible, to establish scientific certainty of causation of many health problems in humans, even though based on laboratory data it is likely that endocrine disruption is involved in a range of human diseases. Because the animal data demonstrate plausible, serious risks to human health, this bias toward false negatives requires the application of the precautionary principle, using animal data as the guide.

Modes of Action

Endocrine disruptors interfere with the functioning of the endocrine system, in at least four possible ways:

- 1) mimic or partly mimic the sex steroid hormones estrogens and androgens (the male sex hormone) by binding to hormone receptors or influencing cell signaling pathways. Those that act like estrogen are called environmental estrogens.
- 2) block, prevent and alter hormonal binding to hormone receptors or influencing cell signaling pathways. Chemicals that block or antagonize hormones are labeled anti-estrogens or anti-androgens.
- 3) affect the synthesis, transport, metabolism and excretion of hormones, thus altering the concentrations of natural hormones.
- 4) modify the making and function of hormone receptors.

However, an understanding of endocrine disrupting processes gets more and more complicated as new research findings are revealed. New compounds, modes of action, LOECs or endpoints are being uncovered on a regular basis. And for every question that is answered, new questions arise. One of the biggest, and probably most complex, mysteries is how substances with different shapes and structures produce similar physiological results.

An example of this is the recent work by Bulayeva and Watson, 2004, where the authors found dissimilar chemicals producing similar effects, at dosing levels never before recorded. The following is from Bulayeva 2004;

“Compounds from different classes of endocrine disruptors with dissimilar chemical structures (e.g., endosulfan as an organochlorine compound vs. nonylphenol as a simple phenolic detergent) can produce the same time-dependent activation pattern for ERKs.”

“There are likely to be specific pathways within the nongenomic signaling network that individual compounds will trigger, leading to different functional end points. Therefore, each xenoestrogenic compound must be tested for an array of possible mechanistic routes of action”.

“Possible reasons for these potent effects not being noted previously are that little testing of the nongenomic pathway has been done, many tests did not examine such low concentrations, and some test conditions probably did not adequately remove endogenous estrogen levels (as we have done by use of low quantities of extensively charcoalstripped serum) to reveal effects of these low concentrations. The potent effects we see on nongenomic signaling mechanisms could explain why concentrations previously determined to be inactive via genomic mechanisms still have toxic and teratogenic effects on wildlife (Brucker-Davis et al. 2001). Therefore, the threat levels of these compounds to wildlife, and probably humans, need to be reconsidered.”

And scientists now believe that harmful effects to signaling pathways is not limited to the endocrine system. Natural chemical signals are important at all levels of life, and any of these chemical signals, in principle, are vulnerable to disruption. As new data are made available, the scope of the problem, and the seriousness of the issues involved is beginning

to emerge.

“Research has clearly shown that EDCs can act at multiple sites via multiple mechanisms of action. Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g., hormone synthesis, transport, and metabolism) have been shown to be equally important. For most associations reported between exposure to EDCs and a variety of biologic outcomes, the mechanism(s) of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects and primary versus secondary effects of exposure to EDCs. It also indicates that considerable caution is necessary in extrapolating from in vitro data to in vivo effects, in predicting effects from limited in vivo data, and in extrapolating from experimental data to the human situation. A collective weight of evidence is essential in determining under what conditions observed effects resulting from exposure to EDCs occur via endocrine mediated mechanisms.”

“Despite an overall lack of knowledge of mechanisms of action of EDCs, there are several examples where the mechanism of action is clearly related to direct perturbations of endocrine function and ultimately to adverse in vivo effects. These examples also illustrate the following important issues:

- a) Exposure to EDCs during the period when “programming” of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals.*
- b) Exposure in adulthood may be compensated for by normal homeostatic mechanisms and may therefore not result in any significant or detectable effects.*
- c) Exposure to the same level of an endocrine signal during different life history stages or during different seasons may produce different effects.*
- d) Because of cross talk between different components of the endocrine systems, effects may occur unpredictably in endocrine target tissues other than the system predicted to be affected”* (WHO 2002, Executive Summary). (A more thorough discussion of mechanisms of action can be found in Chaps 2 and 3 of WHO 2002).

To date, the most widely studied effects are of compounds that simply bind to a hormone receptor and mimic or block normal hormone responses. Receptors are protein molecules that read and respond to hormone signals. This is the effect that initially brought the attention of EDs to the scientific community, and is often the main concern addressed by the public.

Natural hormones travel through the bloodstream and enter cells looking for receptors. Once inside the cell, the hormone binds to a protein receptor (like a key fitting a lock) and forms what is known as a “ligand-hormone receptor complex.” (A ligand is any molecule that binds to a specific site on a protein or other molecule.) Hormones and their receptors have compatible, interlocking shapes, akin to two puzzle pieces or a lock and key.

The binding activates the hormone receptor, which triggers specific cellular processes (like turning a car key which begins the cascading signals necessary to start the engine). The activated hormone receptor then turns on specific genes, causing cellular changes that lead to responses typical of a ligand-hormone receptor complex. In the case of estrogen, for example, these responses can include uterine growth in preparation for pregnancy or prevention of bone loss.

Foreign compounds, although different in shape from natural hormones, can travel in the bloodstream, enter a cell, bind with a receptor and trigger gene expression. It is as if the imposters pick the lock, open the door and fool the receptor into letting them inside. Once bound with the receptor, the mimicker can produce a normal hormone response, cause an abnormal response or elicit no response as it blocks the receptor site and prevents natural hormones from binding.

Though the original scope of inquiry related to endocrine disruption only considered effects produced through an estrogen receptor mechanism, as stated above, this has now been expanded to include the blocking, synthesis, transport, metabolism and excretion of all hormones generated by all organs in the endocrine system. Recent research suggests chemicals that alter hormone production and metabolism may be more harmful and pose greater risk than those that bind hormone receptors (Sharpe 2004).

But the scope of effects and mechanisms of action does not stop there. As research continues to mount about the range of chemical-signaling systems vulnerable to disruption, it is becoming apparent that endocrine disruption is most likely but one example of a broader class of contamination effects, termed "signal disruption" (Fox et al, 2001; McLachlan JA, 2001). All biotic systems use some form of signaling in their reproduction, growth, or other life functions. These chemical signals are important at all levels of organization of life; within cells, among cells, between organs, even between organisms, including from one species to another. Any of these chemical signals, in principle, are vulnerable to disruption. Scientists, for example, have just begun to look at the chemical signals that mediate communication between symbiotic organisms, such as nitrogen-fixing bacteria and the roots of the plants in which they live, and are examining how synthetic chemicals might interfere with these signals. It is through this system of communication that cells talk to each other and produce the results needed to keep a living organism functioning properly. Disrupting these 'signals of life' could have important and far reaching ecosystem impacts.

A simplistic analogy of all this is our information highway, the flow of data through microwave transmissions, the internet, satellites, or phone lines. We know all too well what can happen when these breakdown, when the flow of data is blocked. But what if instead of being blocked, communication was altered to send the wrong signal. What if all the lights turn green at the same time. What if the train is told to continue on the track it's on even though another train is coming from the other direction.

This is the danger that organisms face through signal disruption. The end result is potentially disastrous, biota thrown into chaos. It is with this understanding that risk assessors must view potential effects from EDs. It is not something trivial that can be cast aside with the wave of a few supporting documents. The core of any life form is supported through it's ability to communicate with it's parts, to form a whole. This is adversely affected through "signal disruptors", the full extent of which may not be known for many years to come, if ever. It has been only 15 years since endocrine disruption was first identified as a toxicological concern. In that time, though great progress has been made, it is painfully obvious to the scientific community that much more lies in the realm of the unknown than that which is safely tucked away as scientific fact.

Due to these, and other confounding factors, results from every research project and data review needs to be thoroughly analyzed, through peer review and independent analysis, unlike USDA 2003. There has never been a time when the need for critical, objective analysis has been more important than with the issues surrounding endocrine effects. This is especially true when one considers that new pathways of communication and functional overlap between the various endocrine systems are still being discovered (WHO 2002).

Recent Research on Modes of Action

Recent research has confirmed earlier findings of NP working via non-estrogenic endocrine pathways. As stated above, these modes of action may constitute a greater threat to wildlife and human health than estrogenic mechanisms.

An area of intense interest concerns NPs effect on androgens, steroid hormones such as testosterone that mainly control male traits. They bind to androgen receptors (AR) in a cell, move into the cell's nucleus, and combine with DNA to initiate genetic transcription that leads to androgens bodily effects.

Although weak androgenic NP activity was identified by Sohoni and Sumpter (1998), a more recent study using a yeast two-hybrid system revealed the antiandrogenic effects of NP (Lee et al. 2003). NP may thus have different effects according to the experimental conditions of each assay system. It is therefore also possible that NP exerts a variety of activities under in vivo conditions (Negishi et al 2004).

These findings of antiandrogenic activity were further confirmed in See (2003). In this study, researchers used yeast systems and other laboratory assays to show that nonylphenol (NP) adversely affected the androgen receptor (AR) at many levels including blocking androgen binding, interfering with AR movement into a cell's nucleus, and choking genetic communication. In a yeast assay system, NP inhibited AR interaction with helper molecules that led to decreased enzyme expressions (beta-galactosidase). The decreased activity was more pronounced at higher than lower doses (.1, 1, 10, and 100 micro mole tested) and stronger than a known potent AR antagonist, cyproterone acetate. NP also partially inhibited androgen binding to the AR by 30 percent at 5 nano mole (nM) with irregular doses suggesting a noncompetitive manner (See et al 2003).

In an earlier study, Baldwin demonstrated, after definitive analyses, that both 25 and 100 mug/L 4nonylphenol disrupted components of the testosterone metabolic pathway that would lead to a decrease in the metabolic elimination of testosterone and an increase in the accumulation of androgenic derivatives (Baldwin 1997).

Verslycke found that Mysids exposed to nonylphenol at 10 microg/L had a significantly higher metabolic androgenization ratio. This study indicates that energy and testosterone metabolism of mysids, as endpoints, are able to detect endocrine-disruptive activity of chemicals after short-term exposure to environmentally realistic levels of NP (Verslycke et al, 2004).

Other research has also identified concerns with the synthesis, transport, metabolism and excretion of hormones (Teles et al, 2004, Kullman et al, 2004, Khan et al, 2003).

Kleinow found that transport into bile was significantly reduced with NPE. These results suggest that E2 is a substrate and/or modulator for the catfish biliary pgp transporter, and that NPE potentially influences biliary transport and excretion of E2 (Kleinow et al, 2004.).

There have also been numerous studies that have shown endocrine effects without being able to identify the mode of action.

Due to continuing research that is constantly broadening the scope of mechanisms of action for NPE degradates, it is very important that current risk assessment analysis incorporate recent research. An example can be found in USDA 2003 (Bakke). Here, the only reference to androgen mediated pathways and NP or NPE degradates is found on page 8, where it states *“Exposure to androgens, such as testosterone have been shown to result in an increase in the hepatic production of alpha-2u-globulin in male rats (Murty et al 1987), while the opposite effect can be achieved by exposure to estrogens, such as estradiol (Roy et al, 1977). Therefore a decrease in the incidence of hyaline globules in male rats seen in Cunny et al 1997 may reflect some type of hormonal influence caused by exposure to NP. However, even if the changes to renal hyaline seen in Cunny et al 1997 can be considered to be an indicator of estrogenic effects, this effect was only seen at the highest dose (129 mg/kg/day average dose for males at 2,000 ppm), indicating a NOAEL of 45 mk/kg/day (the average male dose at 650 ppm).”*

As Lee 2003 and See 2003 both clearly show, NP is an antiandrogen, with effects produced at dosing levels 1000 times (or more) lower than stated in USDA 2003.

Toxic Effects and Endpoints Associated with ED and NPEs

The toxic effects and endpoints from endocrine disruption of NPE degradates is a long list. It is also one that is growing longer as the scope of inquiry related to endocrine disruption is broadened to include non-estrogen mimic related effects. These now include androgen blocking, interference with thyroid hormones and progesterone, and many other endocrine related functions. As stated above, as research continues to mount about the range of chemical-signalling systems vulnerable to disruption, it is becoming apparent that endocrine disruption is most likely but one example of a broader class of contamination effects, termed "signal disruption."

Toxic effects from NPE degradates associated with endocrine disruption include

reproductive and developmental toxicity, including embryotoxicity and teratogenicity, genotoxicity, immunotoxicity, neurotoxicity, and cytotoxicity. Research has also shown the potential for these degradates to be mutagenic and carcinogenic, via endocrine pathways, though linkage with these effects is not thoroughly defined.

endocrine disrupting chemicals violate a basic assumption of toxicology and modern risk assessment. For classic toxicants, "the dose makes the poison." Higher doses lead to higher effects. For some endocrine disrupting chemicals, however, effects may disappear at higher levels, or become different qualitatively. New research is illuminating the mechanisms by which these not-so-unusual patterns occur.

Reproductive and Developmental Toxicity

Given the central role that hormones play in guiding the development of the reproductive system and then in controlling its activities once developed, it is not surprising that a major focus of endocrine disruption research has been on reproductive health. There are many studies, especially experimental work with laboratory animals, that document endocrine disruption of the reproductive system. These include reductions in fertility, alterations in sexual behavior, deformations of the reproductive tract and reproductive diseases (reviews of research up to 1999 can be found in WHO, 2002; Environment Canada 2001; Servos, 1999; EU RA 2002).

Human health concerns include; for women, breast and reproductive organ tissue cancers, fibrocystic disease of the breast, polycystic ovarian syndrome, endometriosis, uterine fibroids and pelvic inflammatory diseases. These may be influenced by developmental or chronic lifetime exposure to estrogen mimics.

For men, poor semen quality (low sperm counts, low ejaculate volume, high number of abnormal sperm, low number of motile sperm), testicular cancer, malformed reproductive tissue (undescended testes, small penis size), prostate disease and other recognized abnormalities of male reproductive tissues.

There is also a wide body of data showing other developmental and reproductive effects, including embryotoxic effects associated with NP and NPE degradate exposure (Scott-Fordsmand & Krogh. 2004, Marcial et al, 2003, Kinnberg et al, 2000, Fan et al 2001, Zhang et al 2003a, Kwak et al, 2001, King et al, 2003, Kang et al 2003, Bevan et al 2001, Bevan et al 2003, Bettinetti & Provini 2002, WHO 2002).

Recent Research

Kyselova tested the effect of p-nonylphenol on the body weight, reproductive organ weight and histology, and in vivo fertility of the CD1 outbred mouse strain. The damage to the reproductive organs increased in the F1 generation, when NP influenced the animals during gestation, lactation and the pubertal period. In the group treated by the lower dose of NP, the prostate weight was decreased, and in the group treated by the higher dose, a lower body weight was found. Parental generation males treated with the lower dose of NP had normal spermatogenesis when compared with controls. Interestingly, researchers detected damage to the acrosome in spermatozoa already in the P generation. The acrosomal status (% of acrosome-intact cells) of spermatozoa decreased (compared to control) in the P generation by about 14% (50 µg NP) or 10% (500 µg NP). A marked decrease was observed in the F1 generation: 26% (50 µg NP) and 26% (500 µg NP). Both NP doses had a similar effect on acrosomal damage in the P and F1 generations. The data indicate that we did not find any dose-dependence effect. We can conclude that although spermatogenesis was established in the P generation, NP had an effect on sperm quality. In contrast to the quality, the number of spermatozoa was similar to the control group (in the P and F1 generations). Depending on duration of exposure, there were progressive degenerative changes in the reproductive organs (Kyselova et al, 2003).

Leblanc found a dose dependent increase in the proportion of developmentally compromised neonates, raising the possibility that 4-nonylphenol stimulated egg production without increasing some critical developmental component provided to the eggs by the maternal organisms, such as ecdysteroids, essential fatty acids, or triglycerides. As a result, more offspring were produced, but a significant percentage of the offspring were developmentally compromised, concluding that 4NP's mechanism of embryotoxicity is distinct from that associated with testosterone (LeBlanc et al 2000).

In Mackenzie 2003, gonadal differentiation was observed in leopard frogs (*Rana pipiens*) and wood frogs (*Rana sylvatica*) exposed as tadpoles to nonylphenol. Exposure at micrograms/L concentrations altered gonadal differentiation in some animals by inducing either complete feminization or an intersex condition, and altered testicular tubule morphology, increased germ cell maturation (vitellogenesis), and oocyte atresia. Comparisons between the two species indicate that *R. pipiens* are more susceptible to sex reversal and development of intersex gonads. However, *R. sylvatica* also showed alterations to testicular morphology, germ cell maturation, and oocyte atresia. (Mackenzie et al, 2003)

Immunotoxicity and Neurotoxicity

Since the immune and nervous systems are intricately connected to the endocrine system, there has been a good deal of interest concerning immunotoxic and neurotoxic effects via endocrine pathways. It is, however, a field that is in need of a greater understanding of the mechanisms of action involved (Ahmed 2000, Zala 2004, WHO 2002). This is especially true for immunotoxic effects.

The impact of endocrine disruptors on immune system function and disease resistance is poorly understood. At best we have very preliminary understanding of what may be taking place. There are hints, nonetheless, that this endpoint may have far reaching effects by which endocrine disrupting chemicals undermine human health. Several studies and reviews (see below) indicate that contaminants can erode disease resistance in ways that make people mortally vulnerable to infectious diseases they might otherwise have been able to resist.

If this is the case, then the importance of contaminant effects on health have been vastly under-estimated, because disease statistics would attribute the death to the infectious agent, whereas it would not have occurred without contamination. A new paradigm for studying and preventing many infectious diseases may emerge, in which you need first to understand the contamination history and status of the person exposed to an infectious disease.

The greatest level of current research is focusing on endocrine mediated effects to the developing neural system. During the nine months between conception and birth, the fetal brain is transformed from instructions in genes to a complex, highly differentiated mass of organized cells capable of interacting with the outside world and prepared for learning.

Like virtually all development, the transformation is guided by natural chemical signals instructing cells to differentiate, form brain structures, forge links of immense complexity, and even to die (in a process that is thought to carefully prune unnecessary connections). Normal brain development is heavily influenced by a host of hormonal signalling systems. Thyroid hormones play a major role. The sex steroids (testosterone, estrogen, etc.) contribute to, among other things, sexual differentiation of brain centers, and thereby, to the development of sexual identity and sexual behaviors.

Dependent upon natural hormone signals, neural development is very sensitive to endocrine disruption. What is emerging from research is that brain and behavior are likely to be the most sensitive endpoints vulnerable to endocrine disruption. An important aspect of this research is the realization that small losses in intelligence might have large consequences for a society if they are experienced in a broad swath of the population.

Recent Research

In a recent Negishi study using primary cultured neurons, NP inhibited staurosporine induced neuronal cell death, interfering with caspase-3 activation. NP may, in this manner, disrupt programmed neuronal cell death during development, which would irreversibly lead to an abnormal neural network— including the monoaminergic system—and cause behavioral abnormalities in adulthood, (Negishi et al. 2003).

In a later study, Negishi found that perinatal low-dose NP exposure irreversibly influenced the reception of fear-provoking stimuli (e.g., electrical shock), as well as monoaminergic neural pathways. They also noted a nonmonotonic dose response curve. Dose was 0.1 mg/kg/day (low dose) and 10 mg/kg/day (high dose) orally. The low dose regime showed effects and the higher dose did not (Negishi et al, 2004).

Bevan found that the developing nervous system is exquisitely sensitive to the effects of EDs. Nonylphenol exposure, from early gastrula (Stage 10.5) for approximately 48 hrs (Stage 37) exhibited significant deficits in overall morphology, increased numbers of apoptotic cells (as indicated by TUNEL staining), and dramatic changes in the migration and morphological differentiation of pigment cells derived from the neural crest. In addition, nonylphenol was found to block nerve growth factor (NGF) induced differentiation of spinal cord neurons isolated from early neural plate (Stage 15) embryos and maintained in culture. Taken together, these data indicate that early exposure to NP can induce significant and deleterious effects in the development and differentiation of the nervous system (Bevan et al 2001).

Masuo found that nonylphenol caused a deficit in dopamine neurons, similarly to the deficit caused by 6-hydroxydopamine. Gene-expression profiles after treatment with endocrine disruptors showed variation and differed from those of 6-hydroxydopamine. The results suggest that neonatal treatment with environmental chemicals can generate an animal model of attention-deficit hyperactivity disorder, in which clinical symptoms are pervasive (Masuo et al, 2004).

In early work by Canesi, estrogenic chemicals were shown to affect several vertebrate non-reproductive functions, the immune response in particular. In this work the effects of Nonylphenol on mussel hemocytes were evaluated. The results demonstrate that NP exert in vitro effects on lysosomal membrane stability. When the effects of NP on tyrosine kinase-mediated cell signalling were investigated, estrogenic compounds showed distinct effects on the phosphorylation state of MAPK and STAT members. In particular, NP inhibited p38 MAPK phosphorylation. Overall, the results support the hypothesis that NP may rapidly modulate the function of mussel hemocytes through activation of transduction pathways involving different kinase-mediated cascades. Moreover, the effects of NP on the phosphorylation state of transcription factor STATs suggest that these compounds may lead to changes in gene expression secondary to modulation of kinase/phosphatases. (Canesi et al, 2004).

Iwata examined whether NP can exert direct effects on T cells to suppress or enhance Th1/Th2 development. We used two experimental systems with isolated T cells in vitro. In both systems, 1-10 microM of p-n-nonylphenol suppressed Th1 development and enhanced Th2 development, whereas estrogen by itself failed to affect Th1/Th2 development. These results suggest that p-n-nonylphenol directly suppress Th1 development and enhance Th2 development through mechanisms independent of estrogen receptors, RAR, RXR, PRGR, and GCR (Iwata et al, 2004).

Karrow found that dietary exposure to NP can increase splenic natural killer (NK) cell activity and splenocyte subpopulation numbers in the F(1) generation rats, without similar changes to the F(0) generation. The immunological changes that were observed in the F(1) generation also appeared to be gender-specific (Karrow ET AL, 2004).

Other recent research has found neurotoxicity from NP exposure (Scallet AC 2001,

Scallet et al, 2001, Funabashi et al, 2004, Ohtani-Kaneko 2002)

Cytotoxicity and Genotoxicity

Cytotoxic and genotoxic research of NPE degradates via an endocrine pathway is well documented. Recent research includes Bevan 2001, Bevan 2003, Balasubramanian et al, 2001, Aoki et al, 2004.

Recent research by Teles et al found that a single exposure to NP was able to significantly increase liver P450 content, while its mixture with BNF displayed an antagonistic interference. Considering the xeno/estrogens single exposures, only NP induced an ENA increase; however, the mixture of BNF+NP displayed genotoxic effects. Moreover, a synergism between BNF and NP was thoroughly demonstrated. Fish responses to mixtures of xenobiotics are complex and the type of interaction (synergism/potential or antagonism) in a particular mixture can vary with the evaluated biological response (Teles et al, 2004).

Masuno showed that NP at 5 and 10 microg/ml increased the DNA content by 32% and 68%, respectively, compared with that of the untreated cultures, in which NP was absent during the treatment period. There were many more bromodeoxyuridine (BrdU)-positive cells in the NP-treated cultures, in which NP was present at a concentration of 10 microg/ml during the treatment period, compared to the untreated cultures. These results indicate that NP had the ability to stimulate the proliferation of fully differentiated 3T3-L1 cells. NP at 5 and 10 microg/ml decreased the triacylglycerol (TG) content by 26% and 58%, respectively, and decreased the lipoprotein lipase (LPL) activity by 51% and 71%, respectively (Masuno et al, 2003).

Kudo et al found that the treatment of neural stem cells (NSCs) with 4-nonylphenol for 24 h inhibited cell growth in a concentration-dependent manner. In addition, treatment with 4-nonylphenol resulted in nuclear condensation and DNA fragmentation (morphological changes due to apoptosis) in NSCs after 12 h of exposure, and activated caspase-3 after 6 h and 9 h of exposure. Furthermore, an exposure to 4-nonylphenol led to the accumulation of cells at the G2/M phase interface and down-regulated the protein levels of cyclin A and B1, which are the major regulatory proteins at the G2 to M transition of the cell cycle. Together, these results indicate that, in contrast to other EDs, 4-nonylphenol may exhibit a potent cytotoxicity through apoptosis via the caspase cascade and cell cycle arrest at the G2/M phase, and suggest that 4-nonylphenol may affect neurogenesis in the CNS (Kudo et al, 2004).

Atienzar found that NP and E2 induced some common DNA effects in barnacle larvae and that these specific modifications in RAPD patterns may arise as a consequence of hot spot DNA damage (e.g. DNA adducts) and/or mutations (point mutations or genomic rearrangements). This could help to explain how xenoestrogens mimic the effects produced by natural estrogens. In conclusion, in the field of endocrine disruption, the study of DNA effects induced by estrogens and/or xenoestrogens warrants further investigation. Indeed, changes at the DNA level may be the precursors of some of the numerous effects reported at higher levels of biological organisation such as the feminization of males, developmental abnormalities, and infertility (Atienzar et al 2002).

Ohtani-Kaneko 2002, investigated the effects nonylphenol (1, 10, 100 or 1000 nM at final concentration) on synaptogenesis in primary cultures of fetal rat hypothalamic cells. Results: MAP 2-positive area was increased by the 100 nM nonylphenol treatment, although other concentrations of nonylphenol did not alter the MAP 2-positive area. Nonylphenol also influenced synapsin I-positive area, but in a different manner. A significant increase in synapsin I-positive area was markedly reduced by 100 nM and 1 uM nonylphenol treatments. These results indicate that nonylphenol has different effects on dendritic outgrowth and synaptogenesis. According to the change in synapsin I-positive area, the synaptic density (synapsin I-positive area/MAP 2-positive area) was significantly increased

by 10 nM nonylphenol treatment and decreased by 100 nM and 1 uM nonylphenol treatments. Thus, these results indicate that nonylphenol influences synaptogenesis in primary cultures of fetal hypothalamic cells (Ohtani-Kaneko 2002).

A recent review on data from the National Center for Toxicological Research by Ferguson et al found that *“Recent reviews have focused attention on the need for assessing the neurotoxicity of these compounds following developmental exposure..... Volume of the sexually dimorphic nucleus of the medial preoptic area was reduced by genistein, nonylphenol, and ethinyl estradiol exposure in males.* (Ferguson 2000)

Sato 2002 found that nonylphenol, E2 and other xenoestrogens produced neurotoxic effects at extreme low doses but not through an estrogen receptor mechanism. *“Xenoestrogens are man-made compounds that mimic the actions of estrogens through interactions with estrogen receptors (ERs). Although xenoestrogens have received a great deal of attention as possible causes of brain disfunctions, little information concerning the effects of xenoestrogens on the central nervous system is available. In this study, we investigated the effects of 17beta-estradiol (E(2)) and four xenoestrogens (17alpha-ethynylestradiol, diethylstilbestrol, p-nonylphenol and bisphenol A (BPA)) on the neuronal survival using organotypic hippocampal slice cultures. When the cultured hippocampal slices were exposed to glutamate (1 mM, 15 min), the CA1-selective neuronal damage was induced. Pretreatment with E(2) and the xenoestrogens (24 h) selectively exacerbated the CA3 neuronal damage caused by glutamate. In spite of the marked difference of binding affinities to ERs, all compounds revealed maximal effects at 1 nM. ER antagonists, tamoxifen and ICI 182,780, did not affect responses to E(2) and the xenoestrogens, indicating that these effects are mediated through mechanisms other than ERs.....These results suggest that exposure to E(2) and xenoestrogens during the developmental stage results in a marked influence on synaptogenesis and neuronal vulnerability through mechanisms other than ERs.”* (Sato 2002)

SECTION 2) NON-ENDOCRINE EFFECTS

Acute and chronic toxicity from non-endocrine pathways is just as pronounced as those effects from endocrine mediated pathways. Once again, most research has focused on nonylphenol, though there is a growing trend of using other degradates in testing.

Acute and Chronic Toxicity

USDA 2003 is flawed in its assessment of risk from non-endocrine disruption mediated toxicity, similar to failings found concerning EDCs. Scientifically unrealistic threshold levels are used to establish levels of risk. The following addresses those issues not covered in the endocrine disruption section. Though some of the toxic effects below could be manifested through an endocrine disruption pathway, at this time the mode of action is unknown. Other data used below was also incorporated in the endocrine section (as well as here) because both endocrine as well as non-endocrine effects were reported.

Acute and chronic non-endocrine effects have been shown to occur at similar dose levels as endocrine disrupting effects (Environment Canada 2001, Lussier 1999, Hecht 2002, Zhang 2003, EU RA 2002). There is a large body of data that shows LC50s to occur in the parts per billion range, and acute and chronic adverse toxic effects to occur in the low parts per billion range for NPE degradates. As with endocrine effects, a review of research up to 1999 can be found in WHO, 2002; Environment Canada 2001; Servos, 1999; EU RA 2002. The following brief summaries of findings from Environment Canada's CEPA toxic substances assessment for NPE and metabolites and the European Unions Nonylphenol Risk Assessment.

“There are a large number of studies reporting acute and chronic effects of NP in aquatic biota. There are, however, fewer studies reporting the toxicity of NPEs, and only a few studies that included the NPECs. Although studies described in the literature have used many species, different test methods and

different chemicals, there is a consistent pattern in the toxicity reported. The range of acute toxicity for NP is similar for different organisms: for example, fish (17–1400 Φ g/L), invertebrates (20–3000 Φ g/L) and algae (27–2500 Φ g/L). Chronic toxicity values (No-Observed-Effect Concentrations, or NOECs) for NP are as low as 6 Φ g/L in fish and 3.9 Φ g/L in invertebrates. An acute to chronic toxicity ratio of 4:1 was determined based on the available literature.” (Environment Canada 2001)

The EU RA 2002 has found similar results, though slightly more toxic.

The PNEC (water) is calculated using the assessment factors detailed in the TGD. For nonylphenol short-term and long-term data are available for both freshwater and seawater species for three trophic levels.

*Short-term studies are available for fish, aquatic invertebrates and algae. The most sensitive species appears to be the freshwater invertebrate *Hyalella azteca* with a 96-hour EC of 0.0207 mg/l. Long-term studies are also reported for fish, aquatic invertebrates and algae. The most sensitive species in long-term studies appears to be the freshwater algae *Scenedesmus subspicatus* with a 72-hour EC of 3.3 Φ g/l. As long-term NOECs from at least three species representing three trophic levels are available an assessment factor of 10 may be used. Applying this to the long-term NOEC for algae gives a PNEC (water) of 0.33 Φ g/l.*

For nonylphenol a mesocosm study is available which studied the effects on species from several trophic levels. Generally the effect levels determined in the study for various organisms agree reasonably well with the laboratory data. However, there are several aspects of the experiment design that suggest that the system used, while suitable for detecting gross changes in populations, is not sufficiently sensitive to detect small changes in populations that could become significant with continued exposure. The field study is therefore taken as supporting data in generating the PNEC, but cannot be used as the basis for deriving a PNEC to protect the aquatic compartment.

The PNEC (water) is calculated using all the aquatic toxicity data present on nonylphenol. Data exist indicating toxicity at lower concentrations than the concentrations at which oestrogenic effects are observed. Therefore, the calculated PNEC (water) should be protective for oestrogenic effects in fish as well (EU RA 2002).

As with the risk assessment for endocrine effects in the USDA 2003, the NOAELs incorporated into the risk assessment for acute and chronic non-endocrine toxicity are also unrealistic, do not reflect current scientific understanding of expected effects and misrepresent potential risk from NPE based surfactants. If the USDA 2003 were to incorporate the data used to establish the PNEC in EU RA 2002 into its risk assessment, the MOS would be 33 parts per trillion (ppt) for chronic toxicity and 170 to 200 ppt for acute toxicity, which would be only slightly higher than those the risk assessors should use for endocrine related effects from NPE based surfactants, (though for endocrine disruption there may be no threshold and therefore no way to establish potential risk).

Carcinogenic or Mutagenic Effects (Both Endocrine and Non-endocrine)

Though not listed as carcinogens, NPE and degradates have shown the potential to cause mutations and deformities and are suspected of producing cancer effects through both endocrine and non-endocrine mediated pathways. These concerns should be addressed.

Stated in USDA 2003 on page 18; “To some extent, concern for impurities in technical grade NPE is reduced by the fact that the existing toxicity studies on NPE were conducted with the technical grade product. Thus, if toxic impurities are present in the technical grade product, they are likely to be encompassed by the available toxicity studies on the technical grade product. An exception to this general rule involves carcinogens, most of which are presumed to act by non-threshold mechanisms. Because of the non-threshold assumption, any amount of a carcinogen in an otherwise non-carcinogenic mixture may pose a carcinogenic risk.”

USDA 2003 then states on page 19 “It is important to note that chronic studies involving

NP9E have not determined cancer to be an endpoint in mammals (section 3.1.5)”.

However, USDA 2003 states on page 12; *“In one study, NP9E did induce malignant transformations in BALB/3T3 cells (Long et al 1982), however in another study using the same system, NP9E failed to induce transformations in BALB/3T3 cells (Sheu et al, 1988). In Sheu et al, 1988, the authors suggest that the differences between their results and Long et al 1982 could be due to impurities in the NP9E used in Long et al 1982. Analysis of the impurity 1,4-dioxane showed inducement of malignant transformations (Sheu et al 1988).”*

It appears that USDA 2003 has discounted this potential effect simply because it may have been caused by an impurity. Since this grade of NPE will be used in the IP, this potential effect needs to be incorporated into the risk analysis.

Other recent findings that raise concerns regarding possible carcinogenic or mutagenic effects include;

Seiki et al found that the total incidences of adenomas and carcinomas in the lungs of animals treated with nonylphenol and genistein were significantly higher than in the control group. 5-Bromo-2'-deoxyuridine labeling indices, reflecting cell proliferation, were also significantly elevated in the lungs of rats given 250 and 25 ppm nonylphenol.....These results indicate that nonylphenol and genistein have the potential to promote rat lung carcinogenesis, possibly via a mechanism involving stimulation of cell proliferation and DNA damage caused by oxygen radicals. (Seiki 2003).

Another recent study found that, *“DNA effects include DNA damage as well as mutations and possibly other effects at the DNA level that can be induced by chemical or physical agents that directly and/or indirectly interact with genomic DNA. Not only did exposure to NP and E2 induce changes in RAPD profiles in the exposed barnacle larvae when compared to control patterns, but also, and more importantly, there were similarities in the RAPD modifications in the exposed populations that had been treated to either chemical. We propose that NP and E2 induced some common DNA effects in barnacle larvae and that these specific modifications in RAPD patterns may arise as a consequence of hot spot DNA damage (e.g. DNA adducts) and/or mutations (point mutations or genomic rearrangements)”* (Atienzar 2002).

Zhang et al reported, *“The 96-h EC(50)'s for embryo lethality (arrested egg development) and deformities (curved or unextended shell spines and undeveloped second antennae) were 738 and 263 microg/L, respectively. Reproduction studies were conducted using conditions that stimulate male production (i.e., reduced photoperiod and food levels). An increase in neonate deformities was observed at 50 microg/L (without ethanol), but no changes were observed in fecundity or sex ratios. A decrease in sex ratios was observed at 25 and 50 microg/L (with ethanol) compared to the ethanol control. However, an increase in sex ratios was observed in the ethanol control compared to media controls. The use of ethanol as a solvent carrier confounds the effects of 4-NP on acute toxicity and male production”* (Zhang 2003).

Zumbado 2002 found that, *“These findings taken together suggest that the exposition to alkylphenols induce cell proliferation and spindle disturbances and that these compounds are capable of modulating the expression of putative membrane receptors for estrogens.”* (Zumbado 2002)

Yu 2003 concludes that, *“the test compounds (n-4-nonylphenol, Bisphenol A and dibutylphthalate), like estradiol, markedly enhanced the proliferation of T47D cell and the metaphase of cell division, and the results showed time-dependent and dose-dependent model. These data showed that the tested chemicals could enhance the proliferation of human cervix cancer cell in vitro. This might hint that these chemicals possessed estrogenic activity and they might play their estrogen through estrogenic receptor.”* (Yu 2003)

Fukamachi found that nonylphenol at 10 ppm increased adenocarcinoma and total mammary tumor multiplicity in female Tg rats ($P < 0.05$), but there was no dose dependence, a significant quadratic dose-response trend rather being observed ($P < 0.05$). These results suggest that endocrine disruptors may enhance mammary carcinogenesis, but only in a certain limited dose range under the present experimental conditions. The doses applied, moreover, were all extremely high compared to the possible environmental human

exposure levels (Fukamachi et al, 2004). **Note** (Doses were 10, 25, 100 or 250 ppm nonylphenol. It is assumed that the quadratic curve represented here would be a negative co-efficient, since the lowest dose is mentioned.)

Villanueva found that *“4- NP increased both tumor incidence and latency at 45mg/kg/day. This data further supports a threshold dose of 4-NP of no greater than 45mg/kg/day. None of the mice receiving estradiol formed tumors. In addition, metastasis occurred in two of thirteen mice in the 45mg/kg/day group, but metastasis did not occur in other treatment groups. A 7-day pilot study indicated estradiol levels were increased by 4- NP, but not estradiol or progesterone levels. Therefore, each month mice were tail bled and steroid hormone levels were analyzed. However, steroid hormone levels were not altered significantly by 4-NP. In conclusion, 4-NP induces mammary cancer formation in MMTVneu mice and has a tumor threshold between 30mg/kg/day and 45mg/kg/day in MMTVneu mice”.* (Villanueva et al, 2004).

Other research includes Vivacqua et al, 2003 and Wu F, Safe S, 2004.

3) ANALYSIS OF RISK ASSESSMENTS AND SUPPORTING DOCUMENTS

Dose Response Curves and Low Dose Effects

The key to risk assessment is that the dose makes the poison. However, in the case of NPE degradates and other EDs, finding the threshold dose has proven to be a challenge. The reasons for this are generally accepted to be as;

A) Initially, dosing levels were kept high because no effects were identified. Then scientists realized that EDs often show effects only at extremely low doses, and therefore dosing regimes needed to be lowered. This response curve is known as a non-monotonic dose response curve, and is now well documented and accepted as scientific fact. The presence of non-monotonic dose response curves in endocrine disruption also means that many earlier toxicological tests may have led to erroneous conclusions about safety.

B) Once the dosing regimes were lowered, effects were often seen to occur at all levels tested, therefore no NOEL or NOEC can be established. This can be attributed, in some cases, to the fact that EDs are often times acting as hormone mimics, and are therefore impacting a system that is already at the threshold of effects, i.e. hormones are already present and producing effects.

C) The science of endocrine disruption is new, and the endocrine system and related functions are so complex, that it will probably take many years of detailed research to form a clear picture of all the different types of effects, modes of action and different endpoints involved.

D) Timing of exposure can be a more important factor than rate and duration.

Low Dose Effects

Currently, most research is using dosing regimes in the ppb (parts per billion) range, though some research is now beginning to show that both nonylphenols and octylphenols produce effects in the ppt (parts per trillion) range (Nice 2003, Fent, 2000, Christian and Gillies 1999, Ohtani-Kaneko 2002, Dreze V 2000, Bulayeva 2004, Kwack et al. 2002, Hahn et al. 2002, Uguz 2002, Hemmer MJ, et al. 2002, Ackermann et al. 2002, Pickford KA, et al. 2003, Huang RK, Wang CH, 2001, Czech et al. 2001, Burkhardt-Holm, 2000).

This is a very important fact that has been ignored in current risk assessment protocols used by the Forest Service. As will be analyzed in detail below, USDA 2003 does not use low dose research from NP1EO, NP2EO or NP, justifying this with the assumption that the less toxic carboxylate derivatives are all that would be experienced in an open environment. This is a false and very dangerous assumption. It is also the main reason why

any documentation used for establishing risk from NPE degradates needs to go through a thorough independent peer review.

Once again, reviews of research up to 1999 can be found in WHO, 2002; Environment Canada 2001; Servos, 1999; EU RA 2002. Of special importance to the risk assessment analysis is the EU RA 2002, where the NOEL is 3.3 ppb and the PNEC (Predicted No Effects Concentration [EU risk assessment protocol]) for aquatic species is 0.33 ppb (330 ppt). The PNEC differs from the safety margins used to establish hazard quotients in USDA. The equivalent MOS would be 33 ppt. However, the findings of Bulayeva 2004 would lower the MOS even further, approaching parts per quadrillion range.

In Bulayeva and Watson, 2004, the authors of this study found that xenoestrogens produced time dependent endocrine effects, within 30 minutes of exposure, through pathways that had never been explored before. By analyzing effects to extracellular-regulated kinases (ERKs) in the pituitary tumor cell line, they found that nonylphenols produced effects in the parts per trillion dose range, at the same potency of E2. For sometime, it was assumed that xenoestrogens like NPEs were 1,000 to 10,000 times less potent than E2. However, Bulayeva 2004, and other recent studies have shown that NPE degradates are as potent as E2, and have also shown that they produce negative effects through pathways that E2 does not cause effect through. These new studies have opened up new areas of research for NPEs and endocrine effects. The following is from Bulayeva 2004;

“An important and surprising conclusion from our studies was that all tested estrogenic compounds, except bisphenol A, elicited rapid membrane-initiated actions at very low concentrations compared with their reported potencies in classical genomic pathways (Gutendorf and Westendorf 2001; Hodges et al. 2000; Inoue et al. 2002). All active compounds were able to produce rapid (3–30 min) ERK phosphorylations in the nanomolar concentration range, and some (E2, coumestrol, nonylphenol, and endosulfan) were also active in the subpicomolar range. Compounds from different classes of endocrine disruptors with dissimilar chemical structures (e.g., endosulfan as an organochlorine compound vs. nonylphenol as a simple phenolic detergent) can produce the same time-dependent activation pattern for ERKs...None of the tested compounds was able to precisely repeat the E2 pattern of activation, which may contribute to their disruptive effects on estrogen-mediated endocrine functions.....”

“There are likely to be specific pathways within the nongenomic signaling network that individual compounds will trigger, leading to different functional end points. Therefore, each xenoestrogenic compound must be tested for an array of possible mechanistic routes of action.

Several tested xenoestrogenic compounds (coumestrol, nonylphenol, and endosulfan) demonstrated a bimodal dose–response curve for ERK activation similar to that seen with E2. This is reminiscent of the same bimodal dose–response pattern reported previously for rapid prolactin release after E2 (Watson et al. 1999b) and E2-BSA (Watson et al. 1995) treatment. The reason for this gap in dose responsiveness at intermediate concentrations is still not understood, but it is interesting that other estrogens in the present study demonstrate the same phenomenon. These very low effective doses for xenoestrogens demonstrate that many environmental contamination levels previously thought to be subtoxic may very well exert significant signal and endocrine-disruptive effects, discernable only when the appropriate mechanism is assayed. Possible reasons for these potent effects not being noted previously are that little testing of the nongenomic pathway has been done, many tests did not examine such low concentrations, and some test conditions probably did not adequately remove endogenous estrogen levels (as we have done by use of low quantities of extensively charcoalstripped serum) to reveal effects of these low concentrations. The potent effects we see on nongenomic signaling mechanisms could explain why concentrations previously determined to be inactive via genomic mechanisms still have toxic and teratogenic effects on wildlife (Brucker-Davis et al. 2001). Therefore, the threat levels of these compounds to wildlife, and probably humans, need to be reconsidered.”

Quotes From Recent Low Dose Research

MAP 2-positive area was increased by the 100 nM nonylphenol treatment, although other concentrations of nonylphenol did not alter the MAP 2-positive area. Nonylphenol also influenced

synaptin I-positive area, but in a different manner. A significant increase in synapsin I-positive area was markedly reduced by 100 nM and 1 μ M nonylphenol treatments. These results indicate that nonylphenol has different effects on dendritic outgrowth and synaptogenesis. According to the change in synapsin I-positive area, the synaptic density (synapsin I-positive area/MAP 2-positive area) was significantly increased by 10 nM nonylphenol treatment and decreased by 100 nM and 1 μ M nonylphenol treatments. A significant decrease in the synaptic density was also observed after treatments with 1, 10 and 100 μ M BPA. Thus, these results indicate that nonylphenol and BPA influence synaptogenesis in primary cultures of fetal hypothalamic cells (Ohtani-Kaneko 2002).

Exposure to 50 [μ g/L 4-NP resulted in 100% females considering secondary sexual characters, while external sex-ratio did not statistically differ from unity in control group. In group exposed to 0.5 and 5.0 [μ g/L sex-ratio did not differ from unity but incompletely developed gonopodium was observed in several individuals. Individuals exposed to 50 [μ g/L 4-NP exhibited female or undeveloped gonads, while gonadal sex-ratio did not statistically differ from unity in control group. Percentage of undeveloped gonads increased with 4-NP concentration. Additional observations demonstrated hepatic histopathology in fish exposed to the highest concentration and growth reduction dependent on 4-NP concentration (Dreze V et al 2000).

“Among the APs, 4-t-octylphenol and 4-nonylphenol were found to be considerably more potent than any other compound and estrogenic effects were detectable at 1 and 10 microM, respectively. 4-t-Octylphenol and 4-nonylphenol inhibited the binding of E2 to the ER of MCF-7 cells in a competitive ER binding assay.” (Kwack et al. 2002)

“4-n-nonylphenol contamination caused an inverted dose-response curve. At low test concentrations (1.9-30 microg/l) reduced yolk immunoreactivity occurred, while at medium concentrations (120 and 500 microg/l) no significant effects were observable. In the most highly contaminated group (2,000 microg/l) yolk protein immunoreactivity was elevated to 107% of the control. Female yolk protein contents were affected only in the 3,000 microg bisphenol a/l contaminated group, where yolk immunoreactivity was reduced by ca. 10% compared to the control.” (Hahn et al. 2002).

“All fish died after 4 days of exposure to 660 micro g NP/L. Time-dependent NP bioaccumulation was detected in the tissues of fish exposed to 220 micro g NP/L ($P < 0.05$) and histopathological changes were observed in the livers of fish exposed to 220 micro g NP/L. Furthermore, an increase in the activity of glutathione-S-transferase (GST) was found in the liver of fish exposed to 220 micro g NP/L for 1 week ($P < 0.05$).” “These results indicated that sublethal doses of NP were accumulating in the bodies of the fish and causing histopathological and biochemical changes in the livers of rainbow trout” (Uguz 2002)

“Both chemicals showed a dose-dependent increase in plasma VTG over the entire time course of exposure, with significantly elevated VTG levels by the fifth day of exposure to p-nonylphenol at concentrations of 5.4 μ g/L or greater and to methoxychlor at concentrations of 2.5 μ g/L or greater. Exposure to 0.64 μ g/L p-nonylphenol resulted in highly variable plasma VTG levels of less than 6 mg/ml” (USEPA (Hemmer MJ, et al.) 2002).

“In the chronic study, exposure to NP at 50 μ g/L significantly increased total fecundity and neonate deformities” (Zhang L & Baer KN, 2001).

“However, minor kidney histopathology indicated by increased pyknotic nuclei in kidney tubule and interstitial (hematopoietic) cells was detected at lower estrogenic exposures (≥ 10 microg/l NP nominal) than delayed gametogenesis. Considering all histological parameters in the current study, the rank order of potency for pathological effects in 60 dph zebrafish was 10 ng/l EE > 1 ng/l EE = 100 microg/l NP > 30 microg/l NP > 10 microg/l NP10 (nominal concentrations). Zebrafish from the same cohort examined in the current study that had been placed in clean water from 60 to 300 dph had histologically normal testes and no kidney or liver histopathology. However, increased ovarian follicle atresia was detected at 300 dph in zebrafish exposed developmentally to 100 microg/l NP. Therefore, we conclude that functional rather than morphological changes may be more important for future evaluations of developmental exposure to estrogens in fish, and that negative effects in female rather than male gonads

may contribute to prolonged breeding impairment" (Weber et al. 2003).

"The percentage of males at 60 dph changed from 45% (9/20) in solvent controls to 0% at 10 ng/l EE and 10% at 100 microg/l NP." "Two fish with ovatestes were observed at 100 microg/l NP, while one was observed at 30 microg/l NP. Western blotting showed induction of Vtg at 30 and 100 microg/l NP." "Breeding trials conducted in adult fish from 120 to 160 dph revealed significant reductions in the percent of viable eggs, hatchability, and swim-up success at 10 ng/l EE and 100 microg/l NP. Our results suggest that functional reproductive capacity (breeding success) may be more sensitive than gross morphological endpoints (condition, ovo-somatic index, sex ratio) in adult zebrafish exposed to xenoestrogens during sexual differentiation and early gametogenesis." (Hill RL Jr, Janz DM. 2003).

"The chronic effect of p-nonylphenol on survival and reproduction for two generations of the freshwater cladoceran *Daphnia galeata* was examined by life table experiments. The effects on survival and reproduction were used as the intrinsic rate of natural increase, r , with the Euler-Lotka equation and were analyzed with a simple mathematical model (a power function). The population-level EC(50), the concentration of a substance that reduces the intrinsic rate of natural increase by 50%, was estimated as 65.2 microg/L for the first generation and 81.5 microg/L for the second generation. No transgenerational effect that reinforces adverse responses in the offspring generation has been detected. From a 48-h immobility test an acute LC(50) was estimated to be 60.8 microg/L. The acute LC(50) is a good indicator of the chronic population-level effects of this chemical to this species." (Tanaka Y, and Nakanishi J 2002).

"Nonylphenol, an environmental contaminant, has been shown to induce reproductive abnormalities in male rats.... Nonylphenol was administered orally to male rats at 1, 10 and 100 microg/kg body weight per day for 45 days... The weights of the testes and epididymides decreased significantly whereas the weights of seminal vesicles and ventral prostate remained unchanged at all doses of nonylphenol in treated rats....The results suggest that graded doses of nonylphenol elicit depletion of antioxidant defence system in sperm, indicating nonylphenol-induced oxidative stress in the epididymal sperm of rats". (Chitra KC, et al. 2002)

"The induction of VG and ZRP expression was a more sensitive reaction to the presence of NP than the formation of testis-ova and the reversal of sex. Increased VG expression in trout liver occurred already at 1.05 microg/l NP, whereas VG mRNA levels, quantified by competitive RT-PCR, were not significantly elevated in NP exposed fish. ZRP contents were significantly higher at 10.17 microg/l NP." (Ackermann et al. 2002)

"After exposure to 10 microg NP/l reproduction was impaired as indicated by significantly reduced hatching rates..... The present findings indicate that NP, in an environmentally relevant concentration range, acts as a weak estrogen in directly exposed adult male rainbow trout as indicated by elevated plasma vitellogenin levels. Reproduction success was reduced as indicated by decreased hatching rates. Hormonal imbalances detected in the offspring of exposed fish indicate a transgenerational effect mediated by the endocrine system." (Schwaiger J, et al. 2002)

"Three independent trials were conducted using mortality and burial as endpoints. Amphipod mean lethal concentration to 50% (LC50) was 227 microg/L." (Hecht S, Boese BL. 2002)

"Overall, these results indicate that the lowest-observed-effect concentration (LOEC) and no-observed-effect concentration (NOEC) of 4-NP through the life cycle of the F0 medaka were 17.7 and 8.2 microg/L, respectively. In the F1 medaka, no significant effects were observed on hatching success, posthatch mortality, or growth, but sexual differentiation at 60 d posthatch was affected. Induction of testis-ova in the gonads of the F1 fish was observed in both the 8.2- and the 17.7-microg/L concentrations. The results indicate that 4-NP can have significant effects on reproductive potential of medaka at concentrations as low as 17.7 microg/L." (Yokota et al 2001)

"there was also a significant increase in plasma vitellogenin concentration in the fish exposed via the water to 10 microg/l of 4-NP." (Pickford KA, et al. 2003).

"The LOECs of NP and OP for these events were 11.6 and 11.4 microg/L, respectively. These

results suggest that NP and OP may have adverse effects at similar concentrations during early life stage in medaka. Additionally, we investigated whether the abnormal sex differentiation induced by these alkylphenols would be permanent or reversible once the medaka were returned to clean water. The appearance of the secondary sex characteristics reverted from female to male when fish were returned to clean water. However, gonadal histology showed that intersex gonads still existed, even after the fish were transferred to clean water for two months. These results suggest that the induced feminization of secondary sex characteristics in medaka exposed to alkylphenols during the stage of sexual differentiation may not always be permanent, but the gonadal alteration (testis-ova) may continue much longer." (Seki M, et al. 2003).

"In injection and feeding experiments, vitellogenin levels increased significantly after 14 days. After immersion for 14 and 28 days, respectively, significantly higher concentrations of plasma vitellogenin were detected in male carp exposed to 4 microg/L nonylphenol and octylphenol. When transferred to clean water, the elevated plasma vitellogenin levels in carp exposed to 4 microg/L octylphenol for 42 days returned to control levels within 28 days." (Huang RK, Wang CH, 2001).

"Treatments with TBT and 4-NP (1, 10, 100 ppb) had only slight effects on the egg production of the adults and hatching rate of the eggs. However, increased histopathological changes were observed in epithelial tissues of the adult snails, e.g. lung and foot also characterised by extreme inflammatory processes....The observed histopathological effects due to exposure to tributyltin or 4-NP are suggested to lead to long-term adverse reproductive effects mediated by an impairment of the fitness of the snails. In the experiments the steroid-dependant (beta-sitosterol and t-methyltestosterone) degeneration of the albumen gland caused no obvious adverse effects on the fecundity nor fertility of the adults or on F(1)-generation. However, the impact on fertility following a prolonged exposure to high concentrations of the phytoestrogen cannot be predicted." (Czech P, Weber K, Dietrich DR. 2001).

"The observed responses in survival and reproduction were converted to reductions of the intrinsic rate of natural increase r . The population level EC, which is defined as the exposure concentration that reduces r by 50%, was estimated as 16.1 $\mu\text{g l super}(-1)$." (Tanaka, Y; Nakanishi, J; 2001).

"Nonylphenol is a biodegradation product of nonionic surfactants and has recently attracted considerable attention due to its estrogenic potential. Sexually mature male rainbow trout were repeatedly exposed (one to four periods of 10 days each) to environmentally relevant concentrations of nonylphenol (1 $\mu\text{g/L}$, 10 $\mu\text{g/L}$) and for comparison, trout were injected with estradiol. Since estrogens are known to induce structural changes within the fish skin, a similar effect of xenobiotics with estrogen-like activity was assumed. Samples of skin were evaluated by means of light and electron microscopy and histochemistry. In trout exposed to nonylphenol (1 $\mu\text{g/L}$, 10 $\mu\text{g/L}$) and to estradiol, the structure of the epidermis was altered: an irregular overall architecture was often accompanied by detached pavement cells, vacuolation of the cytoplasm, and severely deformed cell nuclei. However, the granulation pattern of the mucous cells was influenced exclusively after exposition to nonylphenol. The number of large and irregularly shaped mucosomes depended more on the exposure period than on the concentration of nonylphenol. Furthermore, this alteration has not yet been reported for any other pollutant or stressor and, thus, can be classified as an effect that would strongly indicate exposure to nonylphenol." (Burkhardt-Holm, 2000)

"In this study, rainbow trout eggs were exposed after fertilization to NP concentrations of 1 and 10 $\mu\text{g/l}$. Exposure occurred throughout the embryonic, larval and juvenile period under controlled laboratory conditions. After 12 months, induction of VG mRNA was analyzed in the liver by quantitative RT-PCR, and VG protein using polyclonal antibodies in Western blots. The development of quantitative RT-PCR included primer design, competitive PCR using heterologous standards and titration. Both VG mRNA and protein were induced in NP-exposed rainbow trout in a dose-dependent manner. In male fish, increases in VG mRNA and protein were already observed at 1 $\mu\text{g/l}$ NP. This study shows that chronic exposure of fish early life stages to environmentally realistic concentrations of NP leads to induction of vitellogenin." (Fent, 2000).

Data provided by this study suggest that exposure to 1 and 100 $\mu\text{g l}^{-1}$ nonylphenol at Days 7 to 8 post-fertilization results in a change in the sex ratio towards females and an increase in the incidence of hermaphroditism (10 mo later, up to 30% of the resulting adults were fully functional hermaphrodites). Gamete viability is also affected, resulting in poor embryonic and larval development (up to 100% mortality) of the subsequent generation (Nice et al 2003).

Nonmonotonic Dose Response Curve

A significant factor in analyzing research findings and associating these findings to appropriate risk assessment protocol, is the fact that nonylphenol does not follow the typical linear dose response curve currently used by risk assessors. As stated, the non-monotonic dose response curve often seen in nonylphenol endocrine mediated effects forces the re-evaluation of many older high dose studies that found no effects generated. It also questions the validity of current risk assessment protocol, the greater the dose, the greater the effects, and below which exists a threshold of no effects. With NPE degradates and other EDs, even when there appears to be a threshold, until lower doses are tested, there is no certainty that a threshold has been met.

That NPE degradates exhibit non monotonic dose responses with endocrine mediated effects is now accepted as scientific fact (WHO, 2002; Environment Canada 2001; EU RA 2002).

"A key outcome of the (NTP Low Dose Peer Review) was verification that some endocrine disruptors exhibit dose-response relationships described as nonmonotonic, meaning that within a certain dose range, a chemical's effects on a given end point actually become greater as the dose is reduced. The dose-response curves can be shaped like a U, with a high response at both low and high levels of exposure, or like an inverted U, with the greatest response at intermediate dose levels. According to Frederick vom Saal, a professor in the Division of Biological Sciences at the University of Missouri in Columbia, nonmonotonic curves challenge the EPA's standard assumption of linear or threshold dose responses, which holds that toxic effects always lessen as the dose is reduced toward zero" (NIEHS 2001).

The following is from the Commission on Life Sciences, 2000; "Hormonally Active Agents in the Environment";

"Knowing the shape of the dose-response curve for environmental contaminants is critical for understanding how such contaminants...act on organs and organisms. Understanding the dose-response relationship is also critical for the design of studies to test the effects of contaminants.

If an underlying monotonic dose-response function (i.e., a function where response increases as dose increases or at least does not decrease) and a dose below which there is no effect (a threshold dose) are assumed when designing a toxicologic study, there is a risk of failing to understand or properly test a contaminant that does not display a monotonic dose-response function or a threshold dose.

It is well known that some compounds produce nonlinear and even nonmonotonic dose-response functions in some organisms over certain ranges of dose. Furthermore, some compounds can produce different dose-response functions depending on the target organ and the species exposed" (CLS 2000 p82).

"There are numerous examples of nonmonotonic inverted U-shaped dose-response curves from in vitro studies. These studies involve a variety of natural and anthropogenic estrogens (e.g., estradiol, estriol, nonylphenol, and DES), end points (e.g., cell proliferation, prolactin synthesis, and induction of specific mRNAs), and cell lines (e.g., Jordan et al. 1985; Soto et al. 1991; Bigazzi et al. 1992; Pilat et al. 1993; Truss and Beato 1993; Tzukerman et al. 1994; Olea et al. 1996). Sonnenschein et al. (1989) also observed a nonmonotonic response curve for androgen-induced cell proliferation in LNCAP cells by using a diverse group of steroidal and nonsteroidal compounds". (CLS 2000 p110)

The reasons for the nonmonotonic response curve findings are poorly understood at

present. One recognized theory is expressed by Fred vom Saal, the first to document this response in association with EDCs;

"Any endocrinologist will tell you that hormone receptors are up-regulated [stimulated] at low doses and down-regulated at high doses," he says. "In fact, in clinical therapy you can shut down a hormonal system simply by treating with high levels of hormone" (NIEHS 2001).

For risk assessment purposes, it is one more parameter that must be considered. It has been suggested by risk assessors that an inverted U dose response curve actually has two NOAELs and that exposure levels above the curve would have the same effect as those below the threshold. This logic fails on three levels. First, effects are still being generated, they are merely expressing themselves in a different fashion. Second, you reach exposure levels that could produce toxicity through non endocrine disruption pathways. Third, there is no way to gauge exposure levels in the wild.

Recent research highlighting this aspect of dose response includes the following.

Duft found that for NP, there was no clear concentration-dependent response, and therefore, no EC(10) or EC(50) could be estimated, but the data suggest an inverted u-shape type of curve. The LOEC in the experiments with NP was 10 microg/kg (Duft et al 2003).

Hense found that periphyton taxon richness, diversity, and assemblage change was not related to NP concentrations. At the lowest and intermediate concentration, assemblages were significantly different from the controls and the higher concentrations, which were similar during the treatment period (Hense et al, 2003).

Jobling found that the reproductive output response curve in snails exposed to 4-tert-NP was of the inverted U-shaped type. In the snail, the inverted U-shaped response for reproductive output was consistent for the different sampling days for both the effluent and the individual estrogenic chemicals. Chemical hormesis, as this type of response is called, is low-dose stimulation followed by higher-dose inhibition and is now recognised as a type of response to a number of chemicals (Jobling et al 2004).

Other recent research includes Negishi et al, 2004; Ohtani-Kaneko 2002.

In the study cited previously by Bulayeva and Watson, the dose response was an inverted U shape. For NP this showed as effects in the low parts per billion range, no effects produced, and then effects again at the low parts per trillion range.

"The reason for this gap in dose responsiveness at intermediate concentrations is still not understood, but it is interesting that other estrogens in the present study demonstrate the same phenomenon. These very low effective doses for xenoestrogens demonstrate that many environmental contamination levels previously thought to be subtoxic may very well exert significant signal and endocrine-disruptive effects, discernable only when the appropriate mechanism is assayed. Possible reasons for these potent effects not being noted previously are that little testing of the nongenomic pathway has been done, many tests did not examine such low concentrations, and some test conditions probably did not adequately remove endogenous estrogen levels (as we have done by use of low quantities of extensively charcoalstripped serum) to reveal effects of these low concentrations. The potent effects we see on nongenomic signaling mechanisms could explain why concentrations previously determined to be inactive via genomic mechanisms still have toxic and teratogenic effects on wildlife (Brucker-Davis et al. 2001). Therefore, the threat levels of these compounds to wildlife, and probably humans, need to be reconsidered." (Bulayeva and Watson, 2004)

Timing of Exposure

Another important aspect to consider when addressing documentation for risk assessment is the fact that NPE degradates and other EDs have shown a pronounced tendency to exert influence on the endocrine system at different times in the life cycle of all living things. It is imperative that this be incorporated into any BLM risk assessment.

The importance of understanding timing as a risk assessment parameter is that it once again dispels the risk assessment methodology incorporated in USDA 2003. The concept of acceptable dose levels (those below the threshold NOAEL x 100) are only appropriate if a) the most sensitive time for exposure is the tested exposure period and b) these studies are long term chronic or multi-generational studies to identify "later in life" or trans-generational effects. Since nonylphenol has shown itself to produce effects in the very low ppb range during the developmental stage of most organisms that have been tested, this would place the risk quotient multiplier in the ppt, which in turn would place all species at serious risk from exposure.

The following are quotes from a wide range of studies that describe the importance of acknowledging timing of exposure as a risk factor for acute, chronic and multi-generational effects.

"Exposure to EDCs during the period when "programming" of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals" (WHO 2002).

"(T)he effects 1) may be manifested in an entirely different way, and with permanent consequences, in the early embryo, fetus, and neonate from effects as a result of exposure only in adulthood; 2) can change the course of development and potential offspring, with the outcome depending on the specific developmental period(s) of exposure; and 3) are often delayed and thus may not be fully or obviously expressed until the offspring reaches maturity or even middle age, even though critical exposure occurred during early embryonic, fetal or neonatal life" (Colborn et al 1993).

"Another study presented it as, "There are an infinite number of windows of time during embryonic and the early postnatal period when disruption can take place, each leading to potentially different changes in an individual's course of development and behavior. Response to exposure is unpredictable because the process of development is so delicate and complex," (Colborn et al, 1995).

"Concerning the surfactants used by the FS, Lee et al found that there is a critical period of vulnerability to NP during male reproductive development in the neonatal stage. Changes were found when NPs were given to male pups before 13 d of age, but not when given at > or =13 d of age. NP acts on the male reproductive tissues through the estrogen receptor" (Lee 1998).

"It is apparent from research that the main effects from endocrine disruption usually occurs when exposure happens to species developing in the womb or when newly born. However, effects can also occur from exposure later in life. Permanent changes have occurred to animals exposed in adulthood and the potential exists for chronic low level exposure to also affect adult humans" (vom Saal 1993).

"Normal endocrine function is often dependent on cyclical events, rather than steady-state. Timing is everything, as evidenced by significant differences in adverse outcome as a function of age and stage of development" (USEPA).

"Experts suggest that endocrine disruptors pose the greatest risk during fetal development, which is regulated by hormones at specific levels. Hormonal alterations due to maternal exposure in pregnancy could lead to effects such as reduced cognitive function or cancer that might not be evident for months, even years" (NIEHS 2001).

"In trout exposed to nonylphenol (1 mu g/L, 10 mu g/L) and to estradiol, the structure of the epidermis was altered: an irregular overall architecture was often accompanied by detached pavement cells, vacuolation of the cytoplasm, and severely deformed cell nuclei. However, the granulation pattern of the mucous cells was influenced exclusively after exposition to nonylphenol. The number of large and irregularly shaped mucosomes depended more on the exposure period than on the concentration of nonylphenol. Furthermore, this alteration has not yet been reported for any other pollutant or stressor and, thus, can be classified as an effect that would strongly indicate exposure to nonylphenol" (Burkhardt-Holm, 2000).

Sone found that short body length, microcephaly, flexure, edema, and abnormal gut coiling were induced by 20microM NP, BPA or 10microM E2 by 96h p.f. To clarify sensitive stages to these compounds, embryos were exposed to chemicals for 45 or 48h starting at different developmental stages and experiments were terminated 96h p.f. BPA and NP induced abnormalities in developing *X. laevis*, though the sensitive stages of embryos to these chemicals are different, BPA affecting earlier stages and NP affecting at later stages (Sone et al 2004).

Burkhardt-Holm found that the number of large and irregularly shaped mucosomes depended more on the exposure period than on the concentration of nonylphenol (Burkhardt-Holm, et al 2000).

Marcial found that endocrine disruption could occur in copepods following exposure to environmentally relevant concentrations of estrogenic compounds, especially if they are exposed starting from embryonic development (Marcial et al, 2003).

Thibaut found that exposure of ovoviviparous female fish to 4-NP during vitellogenesis and embryogenesis leads to the contamination of the progeny by 4-NP and its metabolites (Thibaut et al, 2002).

Of special note in recent studies exploring the role of timing are the findings of Nice et al. 2003. The authors provide evidence clearly demonstrating that when larvae are exposed to environmentally relevant concentrations of nonylphenol for a single 48 hour exposure at a key stage in their development, long-term sexual developmental effects are induced. Data provided by this study suggest that exposure to 1 ppb and 100 ppb nonylphenol at days 7 to 8 post-fertilization results in a change in the sex ratio towards females and an increase in the incidence of hermaphroditism (10 mo later, up to 30% of the resulting adults were fully functional hermaphrodites). Gamete viability is also affected, resulting in poor embryonic and larval development (up to 100% mortality) of the subsequent generation (Nice et al 2003). This study is important because it is one of the first to identify serious adverse effects from a single "pulse" exposure of extreme low doses with no NOAEL identified.

The USDA 2003 puts much weight in the concept that quick degradation of NPE will limit effects, itself a flawed assumption. However, when one low dose of a substance can produce serious long term effects to both individuals and to populations, it matters not how long something persists, or which degradate is potentially going to be the most common in the environment.

No Threshold

When risk assessors look at potential effects from different dose levels of a toxic substance, they are assuming that the system these chemicals might impact is not carrying a body burden of this substance. If this substance (or other substances that share a common mechanism) is already present in the system, then that is taken into account in an additive fashion. With EDs however, the equation is completely different. Hormone active substances (that is, hormones themselves) are already present in quantities sufficient to cause effect.

In WHO 2002 it was defined as

"The issue of dose–response relationships is perhaps the most controversial issue regarding EDCs. One of the reasons is that EDCs often act by mimicking or antagonizing the actions of naturally occurring hormones. These hormones (often more potent than exogenous EDCs) are present at physiologically functional concentrations, so the dose–response considerations for EDCs are often different than for other environmental chemicals, which are not acting directly on the endocrine system" (WHO 2002).

These principles were first described in Sheehan 1999.

"Risk assessments for nongenotoxic chemicals assume a threshold below which no adverse outcomes are seen. However, when an endogenous chemical, such as 17 β -estradiol (E2), occurs at a concentration sufficient to cause an effect, the threshold is already exceeded. Under these circumstances, exogenous estradiol is not expected to provide a threshold dose".

"There was no apparent threshold dose for E2. A smaller replication confirmed these results. These results provide a simple biologically based dose-response model and suggest that chemicals which act mechanistically like E2 may also show no threshold dose. If so, even low environmental concentrations of such chemicals may carry risk for sex reversal" (Sheehan et al 1999).

Sheehan et al. worked experimentally with sex control in the red-eared slider, a turtle in which sex determination is normally controlled by temperature (via a mechanism in which the hormonal processes involved in sex determination are temperature dependent). They exposed a series of turtle eggs at 28.6°C to a range of doses of 17 β -estradiol. The temperature they chose normally would have resulted in mostly males but some females. They then determined the sex of each egg at hatching. They analyzed the results using a theoretical construct based on the Michaelis-Menten equation, which has been developed in basic chemistry to model enzyme kinetic studies. The data from the large experiment fit the M-M model exceptionally well. The combination of both experimentation and theoretical analysis is very powerful. Their analyses showed that any addition of exogenous estrogen caused a change in the sex ratio of pool of eggs and "that no exogenous estrogen is without risk." This is because in their experimental system, endogenous estrogen is already at a high enough level to exceed the threshold for causing an effect. Endogenous estrogen is already activating the system. A contaminant doesn't have to exceed the threshold because endogenous estrogen already does.

This is an important concept to understand as a risk assessor. Organisms contain substances that put them already past the point of producing effects. The difference from EDs is that the natural hormones are sending the right messages, in the right order, and of the right magnitude to put the proper message into effect. Then they disappear so new messages can be brought forth. An EDC acts on a part of that message stream. It changes the message in a way that makes no sense. Whether or not that message will produce or add to an adverse reaction is dependent on many factors. The fact remains however, that these marauding hormone mimics are causing adverse effects at extreme low doses, often times at all levels tested, with no NOAEL being defined.

Discretionary Use of Supporting Documents

Another important aspect to consider, is that some research projects can be biased. This is especially true when using industry supported documents, such as those produced by the Alkylphenol and Ethoxylates Research Council (APERC).

An example of bias in industry supported research can be found in the controversy that surrounded the early discovery of compounds causing endocrine effects at low doses. When Fred vom Saal published two important bisphenol-A studies from his work group (Nagel 1997, Howdeshell 1999) that showed endocrine effects at environmentally relevant low doses (2ppb), industries response (including members of APERC) to these studies was both swift and damaging. Two industry supported studies attempting to duplicate these findings were quickly introduced. When these two studies were unable to duplicate vom Saal's findings a media barrage ensued claiming that findings of low dose effects by vom Saal were unreplicable and unreliable.

During the height of the controversy surrounding vom Saal's findings, data from the NTP "Low Dose Peer Review" was misrepresented by industry support groups with the intent of discrediting vom Saal's research team. The National Institute of Environmental Health

Sciences was also concerned about industries inaccurate portrayal of the data and stated;

"Although Vom Saal's results were shown to be credible under the panel's statistical reanalysis, they were found not to be reproduced in other, equally credible studies. Interstudy differences in animal strain, diet, dosing regimens, and even housing conditions were all offered as possible explanations for the discrepancy.

Based on the inconsistency of the data, the panelists were not persuaded that a low-dose effect of bisphenol A has been conclusively established as a general or reproducible finding, an admission seized on by the plastics industry, which insists low-dose exposure to the chemical is safe.

"I believe Dr. Vom Saal is convinced of his findings, but he has not convinced his scientific peers," says Paul Foster, program director of endocrine, reproductive, and developmental toxicology at the CIIT Centers for Health Research in Research Triangle Park, North Carolina, a research organization sponsored by industry. *"The inability to reproduce the findings of an increase in prostate weight [or any pathological responses associated with this weight change] in mice [of different strains] and rats indicates that this change is not robust, nor a universal phenomenon likely to have implications for human health risk assessment."*

But Lucier cautions that the panel's statement on bisphenol A shouldn't be taken out of context. Vom Saal's data are of high quality, he says, and the evidence for a low-dose effect can't be discounted. Taken as a whole, the data for bisphenol A and other chemicals reviewed by the panel indicate nonmonotonic, linear, and even threshold responses are all possible outcomes of low-dose endocrine disruptor exposure. The fact that biologic effects were noted in the low-dose region below the NOEL for some data sets, he says, suggests that the EPA should review its current testing protocols to see if changes are required (NIEHS 2001).

Recent research has now shown vom Saal's findings to not only be accurate, but commonplace. And a review of the **industry studies** revealed that it was their own incompetence which led to their failures. **Welshons et al. published** an analysis that showed that the two main industry attempts to replicate vom Saal's work failed because their control animals were inadvertently estrogenized by a contaminant, and thus unable to respond normally to endocrine disruptors (Welshons 2003).

In the last few years, independent laboratories have found results similar to vom Saal's, and these low dose effects are common with NPE degradates (Yokota 2001, Matozo 2003, Tanaka 2001, Tanaka 2002, Zhang 2001, Zhang 2003, Ackerman 2002, Nice 2003, USEPA (Hemmer) 2002, Uguz 2003, Weber 2003, Hill 2003, Hahn 2002, Chitra 2002, Schwaiger 2002, Kwack 2002, Hecht 2002, Servos 1999, Pickford 2003, Seki 2003, Huang 2001, Fent, 2000, Meregalli, 2001, Bevan 2003, Ohtani-Kaneko 2002, Negishi 2004, Fent 2000, Dreze V 2000).

Today, it is routine procedure to use ppb dosing levels in endocrine research. In fact, recent research has shown that, for both octylphenol and nonylphenol, exposure levels in the parts per trillion (ppt) range are, (or could potentially be) producing adverse endocrine effects (Nice 2003, Christian and Gillies 1999, Ohtani-Kaneko 2002, Dreze V 2000). As Sheehan theorizes (see below), for some situations there may be no NOAEL or amount small enough to avoid producing effects.

The use of unbiased data is also important in light of the fact that some of the assumptions reached in USDA 2003 rely heavily on data supplied by APERC. APERC is nothing more than a non-profit public relations firm using industry funded research to counter the overwhelming body of evidence that shows alkylphenols to be anything but "safe for use".

Dose Response Summary

In essence, the "dose-response and threshold" assumptions are the core of any risk assessment. Its use in regulatory science has been a pragmatic step, not something based

on theory or on fact. This assumption is a key part of the way that safety standards are set. All risk assessment must first start by identifying a threshold for effects, or a "no observed adverse effect level" or NOAEL. Then the NOAEL is divided, often by 100. The assumption is that an exposure level calculated in this fashion is safe, and it is used to determine acceptable per day exposure levels.

These fundamental assumptions used to guide current risk assessment are no longer applicable when assessing EDs. Since the issues surrounding dose response to environmental EDs are pivotal to exposure risk assessment and consequently to regulatory considerations, numerous research projects are attempting to come to grips with this need for a new risk assessment model. A review of the state of the science of these concerns was recently published in *Environmental Health Perspectives*. **Welshons et al.** review the issues associated with the underestimation of true bioactivity when only high doses are used in toxicologic studies. The major points considered include low-dose biological activity not observed by traditional testing, nonlinear dose extrapolation, complex receptor responses, and the effects of exogenous exposure on an already active biological pathway. This was their conclusion;

"Information concerning the fundamental mechanisms of action of both natural and environmental hormones, combined with information concerning endogenous hormone concentrations, reveals how endocrine-disrupting chemicals with estrogenic activity (EEDCs) can be active at concentrations far below those currently being tested in toxicological studies. Using only very high doses in toxicological studies of EEDCs thus can dramatically underestimate bioactivity. Specifically: a) The hormonal action mechanisms and the physiology of delivery of EEDCs predict with accuracy the low-dose ranges of biological activity, which have been missed by traditional toxicological testing. b) Toxicology assumes that it is valid to extrapolate linearly from high doses over a very wide dose range to predict responses at doses within the physiological range of receptor occupancy for an EEDC; however, because receptor-mediated responses saturate, this assumption is invalid. c) Furthermore, receptor-mediated responses can first increase and then decrease as dose increases, contradicting the assumption that dose-response relationships are monotonic. d) Exogenous estrogens modulate a system that is physiologically active and thus is already above threshold, contradicting the traditional toxicological assumption of thresholds for endocrine responses to EEDCs. These four fundamental issues are problematic for risk assessment methods used by regulatory agencies, because they challenge the traditional use of extrapolation from high-dose testing to predict responses at the much lower environmentally relevant doses. These doses are within the range of current exposures to numerous chemicals in wildlife and humans. These problems are exacerbated by the fact that the type of positive and negative controls appropriate to the study of endocrine responses are not part of traditional toxicological testing and are frequently omitted, or when present, have been misinterpreted" (Welshons et al, 2003).

Further confounding any attempts to use standard risk assessment methodologies for EDCs is the fact that there are so many variables involved.

"A common dose-response relationship for all effects and for all endocrine disruption mechanisms should not be expected. This conclusion is based on the knowledge that there are many different kinds of hormonal actions of chemicals categorized as endocrine disruptors. These activities include estrogenic, antiestrogenic, antiandrogenic, growth factor modulation, cytokine and thyroid modulation, modulation of hormone metabolism, among many others" (WHO 2002).

The USDA 2003 uses standard dose response methodology to arrive at meaningless hazard quotients that have no basis in current scientific fact. USDA 2003 also uses NOELs from the least toxic degradates (see below). The entire assessment of risk for endocrine effects from NPE degradates is flawed beyond repair and needs to be re-analyzed, re-written and then reviewed by independent experts in the field of endocrine toxicology.

EDCs and Potential Effects to Flora

Recent research has raised a new concern that expands the field of potential adverse effects from EDCs considerably. The study Fox 2001 for the first time looked at signalling pathways outside of the endocrine systems of wildlife. Though this study did not include any alkylphenol ethoxylates, it did include EDCs that function the same as NPEs, as estrogen mimics that bind to the estrogen receptor.

Fox et al. show that EDCs interfere with the ability of nitrogen-fixing bacteria to form a symbiotic relationship with their leguminaceous hosts. This symbiosis is the basis for a key ecological process, nitrogen fixation, which is essential for life on earth.

Rhizobial bacteria form symbiotic relationships with legumes, living in nodules within the plant's roots and converting nitrogen from one chemical form to another. The conversion, called nitrogen fixation, is the principal natural process by which nitrogen is made available for use by living organisms.

The plant-bacteria symbiosis is initiated when the bacterium detects a chemical signal exuding naturally from the roots of the legume. The signals belong to a class of compounds, phytoestrogens, which are so described because of their coincidental ability to interact with vertebrate estrogen receptors.

To detect the signal, the bacteria employs receptors analogous to hormone receptors. The phytoestrogen binds to the bacterial receptor and the resulting complex then activates a gene in the bacterium. Activated, the gene initiates an exchange of chemicals between plant and bacteria that stimulates and maintains the nodules in which the bacteria live.

Fox et al. reasoned that if phytoestrogens were able to interact with the estrogen receptor, then synthetic compounds that interact with the estrogen receptor might be capable of binding with the bacterium's phytoestrogen receptor and reducing gene activation.

Fox et al. worked with alfalfa and its symbiotic bacterium *Sinorhizobium meliloti*. The plant exudes a phytoestrogen, the flavenoid, luteolin, which activates the Nod gene in the bacterium.

They created an in vitro testing system in which they could measure Nod gene induction with luteolin alone and then when a series of endocrine disrupting compounds (EDCs) were added to the experiment.

Nod induction by luteolin at $1\mu\text{M}$ molar concentration was set as the standard for the experiment, or 100% induction. Adding EDCs separately in different concentrations then allowed Fox et al. to determine the potency of EDCs in suppressing Nod induction.

They performed a second set of experiments with alfalfa roots to determine whether the EDC impact on Nod induction would occur in whole organisms. To do this, they inoculated the roots with a bacterium that turns blue upon exposure to one of the biochemical products of Nod gene activation.

Contaminants with estrogenic activity decreased gene expression by up to 90%. In addition to DDT and bisphenol A, methyl parathion, pentachlorophenol and two plant flavonoids (chrysin and genistein) also interfered with phytoestrogen signaling.

There are two important lessons from this study. The first is that it demonstrates conclusively that the symbiosis between legumes and rhizobial bacteria is vulnerable to signal disruption by synthetic contaminants. How extensively this is occurring in the real world becomes an important question, as this symbiosis is crucial to one of the main biogeochemical cycles that makes life on earth possible, the nitrogen cycle.

The second important lesson from this work is that it reinforces the need to consider endocrine disruption as just one type of chemical impact within a broader framework of signal, or message, disruption. Many of life's crucial processes are controlled by chemical signals. Some of these, hormones, mediate events within and among cells, for example, the activation of specific genes. Fox et al. demonstrate that signal disruption can also take place in chemical message systems controlling relationships between organisms, in this case the

two participants in a symbiotic relationship: legumes and rhizobial bacteria.

At the October 2001 hormone meeting at Tulane University in New Orleans, Jennifer Fox and colleagues presented new data from their studies of the impact of EDCs on symbiosis. In this new set of experiments, they exposed growing plants to EDCs and examined the numbers of nodules formed per plant and the mass of the plants. EDCs suppressed nodule number and plant biomass, as predicted by the study reported in Nature (above). Thus the impact of EDCs on Nod gene activation is likely to have real world effects. Currently Fox et al. are working on two similar projects that should be published soon (J. McLachlan pers. comm.).

The question comes to mind, have EDCs already done damage to non-endocrine signalling systems as this research shows possible; and if so, to what extent. Hopefully their results will encourage researchers to begin to look at other chemically-mediated symbioses for signs of chemical disruption.

For instance, scientists have noticed **widespread forest decline** involving trees in Europe and North America. These declines are at least in part associated with changes in the abundance of mycorrhizal fungi, which exist symbiotically with tree roots and are essential for nutrient absorption by tree roots. Investigation into possible disruption of the signals that mediate these symbioses might prove very useful to understand the declines.

INAPPROPRIATE USE OF LEAST TOXIC DEGRADATES IN THE RISK ASSESSMENT FOR AQUATIC SPECIES

Biodegradation and Availability of NPE and Its Degradates

The most glaring failure of USDA 2003 is its use of the least toxic degradates, the carboxylate derivatives of NPE, as the sole compounds of concern in the risk analysis process. This is unsound science at best and a manipulation of the NEPA process and the public trust at the worst.

Below I present an analysis of this issue (USDA 2003 use of the least toxic degradates for risk assessment purposes) that was recently submitted to the R5 NFS as an appeal on the Larson Reforestation Project. If you wish a copy of the Larson DEIS comments referred to below, they were submitted by the environmental organization, Californians for Alternatives to Toxics (CATs) and are available through the R5 NFS.

In CATs Larson DEIS comments, they stated that "NP9E's primary degradates are NP1EO, NP2EO, NP1EC, NP2EC, and NP (Environment Canada 2001)".

The R5 NFS Response to Comments (RoC) replied with "*Your comment is incorrect. Environment Canada, 2001 (EC 2001) actually concludes in Figure 2 of that report that NPE, under aerobic conditions, degrades into NP2EC and NP1EC. The text states that "[biodegradation] involves stepwise loss of ethoxy groups to lower NPE congeners, followed by the production of NPEC and NP, depending upon experimental conditions..." Figure 2 in that report also shows that under anaerobic conditions, NP is formed, and could be formed from NPEC.*" (RoC pM-169)

There was nothing incorrect about CATs statement. What was stated is scientific fact. In referencing Environment Canada at the end of the sentence in question, CATs made no declaration whether the process was anaerobic or aerobic, though had they stated one condition or the other, the statement in question would still have been correct. They were simply stating that which Environment Canada stated on page 19, "*Under aerobic and anaerobic treatment conditions, the biodegradation mechanism involves an initial loss of ethoxy groups, leading to the production of NP1EO and NP2EO and their carboxylate derivatives NP1EC and NP2EC (as well as NPnEC, where n>2, and CAPECs....and CAPEs...*" And though this statement was under the section on wastewater treatment it holds true in almost any situation or condition; in soils, water and wastewater treatment plants.

Also, concerning the quote used in the RoC pM-169 referring to Figure 2 in Environment Canada 2001, and referenced above, the full quote reads as follows;

“...the mechanism involves stepwise loss of ethoxy groups to lower NPE congeners, followed by the production of NPEC and NP, depending upon experimental conditions (Rudling and Solyom, 1974; Maki et al. 1994). The degradation pathway is shown in Figure 2. This pathway is an oversimplification because it does not include NPnEC where $n > 2$ or NPEs with carboxyl groups attached to the nonyl chain. The intermediate and final products of metabolism are more persistent than the parent NPEs, but it is believed that such chemicals will also be ultimately biodegraded. Branching of the nonyl group in NP and NPEs retards biodegradation, as does increase in length of the EO chain” (p18 Environment Canada 2001).

The above quote and the subsequent graphic being referenced, do not support the R5 NFS's contention that under field conditions, the only degradates from NPE based surfactants (R-11) that are of concern to the risk assessment process are NP9E and its carboxylate derivatives. What Environment Canada 2001 is attempting to convey, is that under anaerobic conditions, the pathway primarily includes NPEO congeners and NP. Under aerobic conditions, it is believed that the ethoxylate groups are progressively removed by ether cleavage, forming shorter chained ethoxylates, as occurs with anaerobic degradation. However, a further step in aerobic degradation could be terminal alcohol oxidation followed by cleavage of the resulting carboxylic acid. This is what is meant by the quotation *“...the mechanism involves stepwise loss of ethoxy groups to lower NPE congeners, followed by the production of NPEC and NP.”* The lower congeners of NP9E are NPnEO, where n is < 9 , of which NP1EO, NP2EO are the primary intermediaries. When taken collectively, NP9E's primary degradates are NP1EO, NP2EO, NP1EC, NP2EC, and NP.

It should be noted that both the Maki and the Rudling and Solyom studies cited above (p18 Environment Canada 2001), do not support NP1EC and NP2EC as primary intermediaries for aerobic degradation. The study Maki et al. 1994, is titled *“Degradation of alkylphenol ethoxylates by Pseudomonas sp. strain TR01”*. The key finding in this study is;

“The strain was unable to mineralize Triton –101 but was able to degrade its EO chain exclusively. The resulting dominant intermediate was identified by normal-phase high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry as a nonylphenol ethoxylate with 2 mol of EO units. A carboxylated metabolite, ((nonylphenoxy)ethoxy)acetic acid, was detected by gas chromatography-mass spectrometry.”

In Maki et al.1994, the primary intermediary was NP2EO.

The EU RA stated this concerning the Rudling and Solyom 1974 study, entitled; *“The Investigation of Biodegradability of Branched Nonyl Phenol Ethoxylates”*.

“Rudling and Solyom (1974) studied the degradation of several NPnEO ($n=8, 10$ and 14) using the OECD Screening Test (temperature was 15 or 20 (degrees) C rather than the usual 25 (degrees) C). All three compounds were found to degrade $>90\%$ within 12 days (primary degradation). Gas chromatographic analysis of the test media after 4 days at 20 (degrees) C indicated that NP2EO was the major degradation product and around 50% of this had itself degraded after 28 days. In contrast to this, when incubated at 15 (degrees) C, no further degradation of NP2EO was seen.” (EU RA p 209)

In more recent research similar to Maki et al 1994, John and White found that the carboxylated metabolites were not even formed as an intermediary when using *Pseudomonas putida*. In *“Mechanism for Biotransformation of Nonylphenol Polyethoxylates to Xenoestrogens in Pseudomonas putida”*, the authors found that;

A strain of Pseudomonas putida isolated from activated sewage grew aerobically on the xenoestrogen precursor, nonylphenol polyethoxylate (NPEOx, where x is the number of ethoxylate units) as sole carbon source. Comparative growth yields on NPEOav6, NPEOav9, and NPEOav20 (mixtures with average ethoxylate numbers as indicated) were consistent with utilization of all but two ethoxylate units, and the final accumulating metabolite was identified by gas chromatography-mass spectroscopy as nonylphenol diethoxylate (NPEO2). There was no growth on nonylphenol or polyethylene glycols, and there was no evidence for production of carboxylic acid analogs of NPEOx. Biodegradation kinetics measured by high-pressure liquid chromatography (HPLC) for each component in NPEOx mixtures showed that biodegradation proceeded via successive exocission of the ethoxylate chain and not by direct scission between the second and third ethoxylate residues)” (John and White 1998)

In this study by John and White, as it clearly states above, the final metabolite was NP2EO and no carboxylic acid analogs of NPEOx were produced.

In another study of water and sediment samples from the Kalamazoo River, Michigan, investigators sampled for NP, NP(1-3)EO and NP(1-3)EC. NP and NP1EO were detected, though infrequently, but there were no detections, whatsoever, for any nonylphenol ethoxycarboxylates (NP(1-3)EC) (Kannan K, et al. 2003).

The results from these studies stand in complete contrast to the statement found in the USDA 2003 that only the carboxylic acid analogs of NPnEO, the least toxic intermediaries of NP9E degradation, need be considered in the risk assessment for chronic toxicity for this project.

However, we agree that the wording in Environment Canada 2001 is not precise, as they only state that the first intermediary of NP9E degradation are NPE congeners, before production of the carboxylic acid analogs can occur. For this reason, we would like the FS to address this issue with the use of the supporting document *“Occurrence and Behavior of Alkylphenol Polyethoxylates in the Environment”*, (Montgomery-Brown and Reinhard, 2003). This is a more recent review of the current data of the biodegradation pathways for NPE. The reason for using the latest available science is because *“over the past couple of years, analytical detection capabilities have increased dramatically, allowing the detection and identification of APEO metabolites previously missed. The detection of these metabolites has permitted a greater understanding of APEO degradation in the environment)* (Montgomery-Brown and Reinhard, 2003, p483).

The first point that needs to be understood, is that there is no definitive understanding as to the degradation mechanisms involved with biodegradation of NPE and it's metabolites. This is a critical fact. Studies have produced widely differing results when carrying out similar procedures.

“Despite the fact that the degradation of these compounds has been studied for 40 years, the degradation mechanisms for certain APEO metabolites remain unknown. In fact, one group of metabolites (CAPECs) was not even discovered until 1996 (Ding et al., 1996). Furthermore, although there is no disagreement about the degradability of long-chained APEOs, experimental evidence for the degradation of their metabolites is inconsistent— some experiments indicate that APEO metabolites are readily biodegradable (Staples et al., 1999; Montgomery-Brown et al., 2002), others indicate that they are very recalcitrant (DiCorcia et al., 1998; Shang et al., 1999)”. (Montgomery-Brown and Reinhard, 2003, p477).

“It is generally accepted that APEO biodegradation begins by the rapid stepwise shortening of the ethoxy chain (Fig. 4). Degradation of the branched alkyl chain may proceed as outlined in Fig. 5. Little is known about the biodegradation pathways that lead to the mineralization of APs and carboxylated APEO metabolites (Fig. 6)” (Montgomery-Brown and Reinhard, 2003, p478)

Second, what is known for certain is that the process of degradation usually begins with NP9E being broken down into lower chain NPnEOs. The following is from the EU RA;

“All the available data appear to be reasonably consistent in the findings of nonylphenol ethoxylate degradation during surface water and sewage treatment.

The primary biodegradation of nonylphenol ethoxylates appears to occur rapidly during wastewater treatment, especially with acclimated microorganisms. The first step for NPnEO (where $n > 3$) appears to be rapid removal of the ethoxylate groups to form NP1EO and NP2EO. Once formed these can then be oxidised to form NP1EC and NP2EC or are degraded to nonylphenol or other degradation products where the aromatic ring is broken, leading to complete mineralisation. The NP1EC and NP2EC are then degraded further to mineralisation products (a recent report indicated that these two compounds exceeded 60% theoretical CO₂ generation in 28 days during a OECD 301B (Modified Sturm) ready biodegradation test, but did not fulfil the 10 day window (Williams et al., 1996)).

Under aerobic conditions, oxidation of NP1EO and NP2EO to NP1EC and NP2EC appears to be favoured over formation of nonylphenol. However, under anaerobic conditions, much larger amounts of nonylphenol appear to be formed from NP1EO and NP2EO.” (EU RA Appendix 1, page 220-221)

And from Montgomery-Brown and Reinhard;

“Ethoxylate chain degradation. Degradation of the ethoxylate chain appears to be the first APEO biotransformation process. As seen in Fig. 4, transformation of the parent APEO can occur by two pathways: terminal alcohol oxidation, or ether hydrolysis (Fig. 4, pathways 1 and 2, respectively). Because long-chained carboxylated ethoxylates had not been detected, the primary ethoxylate chain shortening pathway for under both aerobic and anaerobic conditions, was believed to be stepwise ether hydrolysis accompanied by the loss of ethylene glycol (Fig. 4, pathway 2). This process proceeds rapidly until short-chained APnEOs ($n = 1-3$) are formed. As the ethoxy side chain is shortened, APEO metabolite solubility decreases and their persistence increases. Under anaerobic conditions short-chained APnEOs ($n = 1-3$) are converted to APs (Fig. 6, pathway 8); under aerobic conditions, they are oxidized (Fig. 4, pathway 1) (Thiele et al., 1997; Maguire, 1999). Carboxylation increases both the solubility and persistence of these metabolites (Ahel et al., 1994b)” (Montgomery-Brown and Reinhard, 2003, p479).

However, this is not always the case.

Recently, Jonkers et al. (2001) concluded that the dominant pathway in aerobic APEO biodegradation is the oxidation of the terminal alcohol on the ethoxy side chain (Fig. 4, pathway 1), not, ether hydrolysis. This conclusion was made because long-chained (up to $n = 15$) APnECs were observed almost immediately upon beginning their experiment. Stepwise shortening of the carboxylated ethoxy chain might proceed by terminal ether hydrolysis (Fig. 4, pathway 3). Because only small amounts of AP2EO were detected, it is plausible that the APEOs observed in the environment are produced in anaerobic microenvironments. The researchers were only able to account for 19% of the initial APEO—this suggests either complete APEO mineralization or the formation of undetected metabolites”(Montgomery-Brown and Reinhard, 2003, p480).

It should be noted that the Jonkers et al 2001 study did not show conclusively that the primary degradates were carboxylate derivatives as only 19% of the initial compound were recovered. Since mineralization of NPE metabolites often occurs very slowly (if at all), we can only assume that the 81% that was unrecovered was in the form of undetected metabolites.

What should become obvious from all of the above is that the environmental fate of NP9Es can not be clearly defined. This is why CATs suggested that the R5 NFS was using poorly understood data to arrive at the assumptions being used in the risk assessment, that chronic toxicity values should be based on the NP(1-2)EC metabolites (Larson FEIS p III-22). The supporting document for this assumption is USDA 2003. On page 23 of USDA 2003 it states;

"In aerobic conditions, NP9E, and other NPEs, are broken down through the removal of ethoxylate groups as a result of microbial action or photolysis, into shorter-chain ethoxylates (John et al 2000; John, White 1998; Maki et al 1996; Castillo et al 2001; Manzano et al 1998). Some studies show this breakdown resulting in formation of short-chain NPE (NP1E or NP2E) (John et al 2000; Tanghe et al 1999, Castillo et al 2001), however most studies indicate that further reactions cause formation of short-chain nonylphenol ether carboxylates (NP1EC, NP2EC) (Ahel et al Page 24 1994a, 1994b; APERC 1999a; Di Corcia et al 1998; Jonkers et al 2001; Manzano et al 1998 and 1999; Maguire 1999; Maki et al 1996; US EPA 1996)".

This analysis is misleading, when it states *"most studies indicate that further reactions cause formation of short-chain nonylphenol ether carboxylates"*. First, this sentence is not clearly defined. Is this statement meant to convey the assumption that all NPnEOs are expected to be converted to NPnECs. And if so, what is the lag period expected to be when first converting from NP9E to *"shorter-chain ethoxylates"*, and then converting to *"formation of short-chain NPE (NP1E or NP2E)"*, before *"further reactions cause formation of short-chain nonylphenol ether carboxylates (NP1EC, NP2EC)"*. Since timing of exposure is a critical element in risk assessment analysis, the above needs to be clearly defined and analyzed.

Second, some of the studies cited above as supporting documents in USDA 2003 p 23, show the carboxylate derivatives NPnEC as a step in the chain (with conversion occurring from NP(1-2)EOs) and in varying degrees. Others do not, they merely suggest it as a possibility. APERC 1999a is an industry supported review that simply states *"During APE biodegradation, microbial organisms remove ethoxylate groups from the end of the ethoxylate chain. This may be accomplished by conversion of the end of the ethoxylate chain to a carboxylic acid known as an alkylphenol ether carboxylate (APEC), which then is further degraded. The process of ethoxylate chain degradation continues until one or two ethoxylate units remain. At this stage in the biodegradation process, the phenol "ring" of the APEs and APECs is degraded"*. Only Jonkers found that long chained ethoxylates could be converted to long chained carboxylates.

Ahel 1994a shows that effluent entering a wastewater treatment plant contained 94% NPnEOs and only 3% NPnECs. After final treatment, based on analysis of the various effluents and sludge in the plants, the totals were; 25% NP, 19% NPnEOs, 19% NPnECs

Ahel 1994b found a decrease in NPnEOs and an increase in NPnECs in water samples, and an increase in NP in sediments.

As stated, Jonkers found primarily NPnECs, but these accounted for only 19% of the total NPE solution with 81% being unknown derivatives.

Manzano et al 1999 found that carboxylation occurred only after NP(1-2)EOs had been formed

Di Corcia found CAPECs to be a predominant intermediary. It was also found that oxidation primarily occurred from shorter chain ethoxylates. This was discussed in Tanghe 1998;

"However, w- and b-oxidation of the alkyl chain only occurs when it is not highly branched (Osburn 1966) or when dealing with a linear alkyl chain (Corti 1995). Because commercially available

NPnEOs have highly branched nonyl chains which prevent the β -oxidation, the primary degradation of these surfactants is assumed to start at the ethoxy chain. Nevertheless, characterization of intermediates from biotransformation of branched NPnEOs has shown that compounds having both side chains (alkyl and ethoxy chains) oxidized can occur, but were presumably generated from less extensively branched isomers (Di Corcia 1998)" (Tanghe et al 1998).

As USDA 2003 states, there is a shortening of the ethoxylate chain. As stated in the EU RA, Montgomery-Brown and Reinhard, 2003, and Tanghe 1998, the chain is usually shortened to NP2EO or NP1EO, which are highly toxic substances and more persistent than the parent compound. Whether they would be further degraded to NP1EC, NP2EC, NP, CAPECs, brominated derivatives, chlorinated derivatives, lesser known metabolites, or carbon dioxide, inorganic salts and water is dependent on many factors. Since Jonkers et al 2001, showed only 19% recovery of the NPE solution, the possibility of the presence of unknown metabolites with unknown toxicity levels is very real. It is also possible that under certain conditions, terminal alcohol oxidation may occur in the primary stages of degradation, producing carboxylate derivatives without first forming shorter chain NPEOs. Irrespective of which direction the pathway takes, its path most often begins with a shortening of the ethoxylate chain, usually resulting in production of NP(1-2)EOs before branching further.

The important thing to consider in risk assessment protocol for NP9E and its degradates, is that as NP9E degrades, it most likely branches off into degradates that are more toxic and more persistent. This process of degrading to shorter chain ethoxylates can begin immediately, as NP9E has shown that it is readily biodegradable upon release into the environment. The immediate degradates however have shown themselves to be more persistent and have shown themselves to produce acute, chronic and endocrine effects at environmentally relevant doses. Endocrine effects are especially troubling as they can produce effects when exposure occurs at a particular time. It doesn't take days or weeks, if the host is at a stage of development that provides an open window for endocrine effects to be produced, the damage is done.

Skewing Data to Establish Unsubstantiated Toxicity Values used for Risk Assessment

There is, however, no body of data that would lead one to conclude that the only compounds in the degradation process that need be considered are NP9E and NPnECs. Yet this is exactly the leap that the USDA 2003 takes. From the poorly defined statement found on page 23 of USDA 2003 and quoted above, this conclusion is then reached;

"The potential for effects on aquatic species are based on estimated concentrations of NP9E or NP1-2EC in water that are identical to those used in the human health risk assessment. The estimated rate of contamination of ambient water associated with the normal application of NP9E is 0.0125 mg a.e./L (12.5 ppb). For acute exposure scenarios, the highest estimated concentration of NP9E in water after an accidental spill is about 6.1 mg a.e./L (ppm) with a range of about 3.0 to 15.1 mg a.e./L. As another exposure scenario, if the Forest Service were to overspray an herbicide mixture with an 80% NPE-based surfactant into a small pond or stagnant stream reach, with no foliar interception, instantaneous levels of NP9E could approach 1.5 mg/L (1,500 ppb) and the concentration of NP and the short-chain ethoxylates (NP1E and NP2E) could approach (0.075 mg/L (75 ppb) (refer to worksheet 1 in Appendix 1). Assuming a more realistic live stream, these levels would be quickly lowered as water is mixed through stream flow.

As discussed in section 3.2.3.3, the breakdown of NPE would likely not liberate NP, and any free NP in the surfactant would be broken down in the forested environment or bound to soil particles.

Therefore, it is very unlikely that NP would be found in forest streams above the level that might be found in the NP9E mixture originally. As stated in section 4.3, the acute toxicity of NP9E includes this small percentage of NP and short-chain NPEs, so no adjustment for acute exposures is necessary.

Based on environmental fate, the toxicological compound of interest is more likely to be the short chain NPECs (NP1EC, NP2EC), as they will be formed in the forested environment and their persistence would make them more available for aquatic wildlife exposure and for exposure to terrestrial wildlife through water consumption. As stated in section 3.2.3.3.2, the assumed levels of NP1-2EC in water will be based on water monitoring and set at 0.007 mg/L (with a range of 0 to 0.014 mg/L)" (USDA 2003, page 51) .

Currently, USDA 2003 uses the acute toxicity values for NP9E for establishing risk of acute toxicity, and chronic toxicity values for NPnECs for establishing risk of chronic toxicity.

These are not valid parameters for assessing risk from NPE based surfactants. Choosing to analyze risk by isolating and referencing only the least toxic forms of NP9E degradation and ignoring more toxic derivatives is not sound science. In essence, it is nothing more than skewing data to produce the desired results. Since the whole risk assessment analysis is based on flawed toxicity values, the entire risk assessment for this project must be considered invalid. The difference in toxicity between NP2EO and NP2EC is quite dramatic. Maki *et al.* (1998) measured 48-hour LC50s in *Daphnia magna* for NP2EO (115–198 µg/L) and NP2EC (990 µg/L). These data suggest that the NPnECs are much less toxic than the corresponding NPnEOs.

Acute toxicity usually incorporates a time span up to 96 hours. Within the first 96 hours after release of NPE based surfactants, much of the NP9E can have already broken down into shorter chain ethoxylates (NPnEO). For this reason, the acute toxicity values of NP(1-2)EO is a more relevant reference for acute toxicity risk assessment. When not available, acute toxicity values for NP should be used, as the toxicity of NP(1-2)EO has been shown to be only slightly less than NP (Environment Canada 2001).

Chronic toxicity usually incorporates periods of 30 days or more. The shorter chain ethoxylates (NP1-2EO) have shown that they are moderately persistent, as stated in EU RA 2002 and Environment Canada 2001. Therefore, these compound's chronic toxicity values, or once again NP, should be used for establishing risk from chronic toxicity as well.

However, since NP9E and its metabolites have shown that they are endocrine disruptors, and since endocrine effects can be generated from extreme low doses by these metabolites (1 ppb as stated above), toxicity values for endocrine effects, where applicable (i.e. in early stages of development), should be used as reference for aquatic species.

Importance of Assessing Risk from the Full Mixture of Degradates.

The predicted environmental concentrations for NPEs used in USDA 2003 are also skewed. USDA 2003 states that "As stated in section 3.2.3.3.2, the assumed levels of NP1-2EC in water will be based on water monitoring and set at 0.007 mg/L (with a range of 0 to 0.014 mg/L)" (USDA 2003, page 51) .

The relevant data from this section referenced are;

3.2.3.3.2. Longer-Term Exposure

There has been no monitoring of NP9E or its metabolites in forested environments. There is considerable monitoring data that can define 'background' levels of contamination - i.e., levels in water

that are not associated with specific applications of NP9E. Although these monitoring studies are generally associated with downstream waters, they can provide a conservative estimate of levels of NP9E and metabolites.

Sampling in sites removed from sewage treatment plants and mills are more representative of potential background levels in forests. Bennie, 1999, compiled data from sampling studies in Canada and the U.S. This monitoring showed that NP3-17E ranged from <1.6 to 14.9 µg/L. NP1EC ranged from nondetectable (ND) to 2 µg/L and NP2EC ranged from ND to 12 µg/L. NP levels ranged from ND to 2 µg/L in river water and from <0.002 to 72 µg/g (dry weight) in sediments (Bennie 1999). NP1E ranged from <0.02 to 7.8 µg/L in water and from <0.002 to 38 µg/g in sediments. NP2E ranged from <0.02 to 10 µg/L in water and <0.015 to 6.0 µg/g in sediments. The highest levels reported in Bennie 1999 for NP, NP1E and NP2E were from Hamilton Harbor, Ontario, downstream from the discharge of a sewage treatment plant.

For chronic exposure scenarios involving water, the cumulative levels of NP1-2EC as derived from Bennie 1999 will be used as the upper level (14 µg/L), with zero being the lower level and the midpoint of 7 µg/L being the central value.

This analysis is both confusing and illogical, and raises many questions. First, the entire risk assessment is based on the assumption that only NPnECs will be present in appreciable quantities. Yet the data used to establish these exposure concentration scenarios for NPnECs, (Bennie 1999), clearly shows that NPnEOs will be present in greater concentrations in river water than the NPnECs. Using the data above, the cumulative totals from Bennie 1999 are as follows; NP = ND to 2 ug/l, NP(1-2)EO = <0.04 to 17.8 ug/l, NP(3-17)EO = <1.6 to 14.9 ug/l, and NP(1-2)EC = ND to 14 ug/l. This further acknowledges that which has already been shown to be true, that NPnECs may not be the primary metabolites in need of assessing.

It should be noted that, irrespective of where the highest levels for NP(1-2)EO are recorded, (stated above as downstream from a STP), these findings are still significant since both primary and secondary treatment in a STP tend to convert NPnEOs to NPnECs (Ahel, 1994, USDA 2003, Di Corcia 2000). It should also be noted that none of these sites in Benny 1999 were directly downstream from a herbicide spray project, nor were they low flow streams, or standing bodies of water, that had just received a fire chemical drenching.

Second, as clearly stated in Environment Canada 2001, it is not the levels of an individual NPE degradate that needs to be considered in the assessment of risk, but the full mixture, since all components in the biodegradation pathway are more toxic than the parent compound. The following are quotes from Environment Canada 2001;

“Because NPEs occur as complex mixtures in the environment and have different toxicities and estrogenic potencies, the approach used in this assessment was to first assess each chemical separately, then assess the complex mixtures found in the environment” (Environment Canada 2001 p 42).

“In one study, NP2EO and NP1EC were only slightly less potent than NP in inducing vitellogenin in trout hepatocytes. NP, NPEs and NPECs are found as complex mixtures in effluents, and their combined estrogenic effects on aquatic organisms should be considered together..... Estrogenic responses occur at concentrations similar to those at which chronic toxicity occurs, although biochemical and histological changes have been reported at concentrations a factor of 10 lower” (Environment Canada 2001 p 2).

“The relative estrogenic potency determined in several different in vitro systems is in the order NP >NP1EO = NP2EO > NP1EC = NP2EC > NP9EO. The estrogenic responses appear to be at least additive and should, therefore, be considered as a group” (Environment Canada 2001 p 28).

“It is important that all of the NPE metabolites, not only NP, be considered together to assess the

potential for impacts in the environment” (Environment Canada 2001 p 2).

“In addition to examining the exposure and toxicity of each metabolite individually, 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 overall potential risk of the group” (Environment Canada 2001 p 46).

“As observed in field measurements, NP and NPEs occur as complex mixtures, and the toxicities of the metabolites are expected to be additive. When NP is considered alone, only three sites have predicted concentrations in receiving waters that exceed a value of 1 µg/L. When NP1EO and NP2EO are considered in addition to NP, an additional four sites exceed the ENEV” (Environment Canada 2001 p 57).

It is basic sound science to incorporate the full mixture of NPE degradates as a single concern. The endpoints and mechanisms of action for toxic effects are basically the same for all the primary intermediaries. It is also basic sound science to use the acute, chronic and endocrine toxicity values for NP(1-2)EO, and where these are not available, NP, as the references for establishing risk.

The full mixture totals, adding NP, NPnEOs and NPnECs, that can be derived from Benny 1999 are 2 ug/l to 48.7 ug/l. Since the full mixture should be assessed, the central value is the full concentration of degradates, which is 48.7 ug/l. This is the total amount of toxic compounds related to the application of NPE based surfactants that aquatic species could be exposed to at any one time. The upper level would have to be greater than 48.7 ug/l.

Conclusion

Because of the acute, chronic and endocrine toxicity associated with NPE and its degradates, it is imperative that a more thorough analysis be performed. Accurate NOEL's must be established from the full body of data relating to NPE toxicity, and not just those studies that tend to support an argument, as found in USDA 2003. Aquatic NOELs should be in the parts per trillion range. Risk quotients need to reflect the low dose figures found in endocrine research.

It is only through a rigorous analysis of ecological effects that land managers can arrive at a sufficient understanding of the different effects each chemical might have on the whole of the environment.

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