WHITE PAPER

AQUATIC LIFE CRITERIA FOR CONTAMINANTS OF EMERGING CONCERN

PART I

GENERAL CHALLENGES AND RECOMMENDATIONS

Prepared by the
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DRAFT DOCUMENT

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List of Acronyms:

ACR	Acute to Chronic Ratio
ALC	Aquatic Life Criteria
Ah	Aryl Hydrocarbon (receptor)
AV	Acute (toxicity) value
AWQC	Ambient Water Quality Criteria
CCC	Criterion Continuous Concentration
CEC	Contaminants of Emerging Concern
CMC	Criterion Maximum Concentration
CV	Chronic (toxicity) value
CWA	Clean Water Act
CYP	Cytochrome enzymes (P450)
EDC	Endocrine Disrupting Chemicals
E2	Estradiol (natural estrogen)
EE2	Ethynylestradiol (synthetic pharmaceutical estrogen)
ELS	Early Life-Stage (toxicity test)
EPA	Environmental Protection Agency
FAV	Final Acute Value
FACR	Final Acute to Chronic Ratio
GMAV	Genus Mean Acute Value
GMCV	Genus Mean Chronic Value
HPG	Hypothalamic-Pituitary-Gonadal (axis)
HPT	Hypothalamic-Pituitary-Thyroid (axis)
LOEC	Lowest Observed Effect Concentration
MDR	Minimum Data Requirement
MOA	Mode of Action
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Development and Cooperation
OWC	Organic Wastewater Contaminant
PBDE	Polybrominated Diphenyl Ether
PPCP	Pharmaceutical and Personal Care Product
POP	Persistent Organic Pollutant
SMAV	Species Mean Acute Value
TBT	Tributyltin
USGS	United States Geological Survey
VTG	Vitellogenin protein
vtg	Vitellogenin gene transcript
WWTP	Wastewater Treatment Plant

1.0 INTRODUCTION

Under the United States Clean Water Act (CWA) (33 U.S.C. Sections 1251-1387), EPA is required to take a number of actions to protect and restore the ecological integrity of the Nation's water bodies. Under Section 304(a) of the CWA, EPA must develop and publish ambient water quality criteria. Ambient water quality criteria (AWQC) are levels of individual pollutants, water quality characteristics, or descriptions of conditions of a water body that, if met, should protect the designated use(s) of the water. Examples of designated uses of a water body include swimming, drinking water, fishing, fish spawning, and navigation. States and authorized tribes establish designated uses for their water bodies. AWQC are recommended guidance that states and tribes may use as part of their water quality standards to protect water bodies for their designated use from chemical pollutants.

AWQC for aquatic life (aquatic life criteria, ALC) developed under Section 304(a) reflect the "latest scientific knowledge" concerning "all identifiable effects" of the pollutant in question. These criteria are based solely on data and scientific determinations on the relationship between environmental concentrations of the pollutant and its effects. Criteria do not consider social and economic impacts, or the technological feasibility of meeting the chemical concentration values in ambient water. Since the early 1980's, EPA has been developing ALC to protect aquatic organisms from chemical specific pollutants under Section 304(a) of the CWA. In 1985, EPA published *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses* (hereafter referred to as the "*Guidelines*"; Stephan et al. 1985). The *Guidelines* has provided uniformity and transparency in the derivation methodology of ALC for a large number of compounds among several classes of chemicals. The majority of EPA's currently recommended ALC have been derived using the methods outlined in the *Guidelines*.

While the *Guidelines* remain the primary instrument the Agency uses to meet its broad objectives for the development of ALC, there have been many advances in aquatic sciences, aquatic and wildlife toxicology, population modeling, and ecological risk assessment that are relevant to deriving ALC. Some of the advances have been addressed through supplemental guidance on the derivation or site-specific modification of criteria (Prothro 1993; U.S. EPA 1994a), while others have been incorporated directly into derivation of individual ALC for certain chemicals (e.g., saltwater chronic aquatic life criterion for tributyltin, U.S. EPA 2003). Recently, considerable attention has been generated by a widely ranging group of chemicals termed, in this document, contaminants of emerging concern (CECs). As is discussed in the body of this document, some of these CECs present challenges for the application of the *Guidelines* to ALC development.

1.1 What is a Contaminant of Emerging Concern?

The term "contaminant of emerging concern" is being used within the Office of Water to replace "emerging contaminant," a term that has been used loosely since the mid-1990s by EPA and others to identify chemicals and other substances that have no regulatory standard, have been recently "discovered" in natural streams (often because of improved analytical chemistry detection levels), and potentially cause deleterious effects in aquatic life at environmentally

relevant concentrations. They are pollutants not currently included in routine monitoring programs and may be candidates for future regulation depending on their (eco)toxicity, potential health effects, public perception, and frequency of occurrence in environmental media. CECs are not necessarily new chemicals. They include pollutants that have often been present in the environment, but whose presence and significance are only now being evaluated.

CECs include several types of chemicals:

- Persistent organic pollutants (POPs) such as polybrominated diphenyl ethers (PBDEs; used in flame retardants, furniture foam, plastics, etc.) and other global organic contaminants such as perfluorinated organic acids;
- Pharmaceuticals and personal care products (PPCPs), including a wide suite of human prescribed drugs (e.g., antidepressants, blood pressure), over-the-counter medications (e.g., ibuprofen), bactericides (e.g., triclosan), sunscreens, synthetic musks;
- Veterinary medicines such as antimicrobials, antibiotics, anti-fungals, growth promoters and hormones;
- Endocrine-disrupting chemicals (EDCs), including synthetic estrogens (e.g.,17α-ethynylestradiol, which also is a PCPP) and androgens (e.g., trenbolone, a veterinary drug), naturally occurring estrogens (e.g.,17ß-estradiol, testosterone), as well as many others (e.g., organochlorine pesticides, alkylphenols) capable of modulating normal hormonal functions and steroidal synthesis in aquatic organisms;
- Nanomaterials such as carbon nanotubes or nano-scale particulate titanium dioxide, of which little is known about either their environmental fate or effects.

1.2 Why is EPA Concerned with CECs?

The variety of chemicals labeled as CECs leads to a variety of concerns for EPA. Widespread uses, some indication of chemical persistence, effects found in natural systems, and public concerns have made clear the need for EPA to develop criteria that can be used to help assess and manage potential risk of some CECs in the aquatic environment. A recent U.S. Geological Survey (USGS) reconnaissance study (Kolpin et al. 2002) provides a good example of the prevalence of a wide range of CECs in U.S. streams. Improved analytical chemistry techniques were used to document the occurrence of what the authors called organic wastewater contaminants (OWCs) being released into surface waters from wastewater treatment plants (WWTPs). The targeted OWCs included PPCPs, veterinary medicines and other EDCs. The investigators found at least one of 95 different target OWCs in 80 percent of the 139 streams sampled. A median of seven, and as many as 38, OWCs were found in single samples.

The use and occurrence patterns associated with CECs are varied. Some CECs are similar to conventional toxic pollutants in that they are associated with industrial releases, whereas many others are used by the general public every day in homes, on farms, by businesses and industry (Daughton and Ternes 1999). PPCPs acting as EDCs can be released directly to the environment after passing through wastewater treatment processes, which are typically not designed to remove these pollutants from the effluent (Halling-Sorensen et al. 1998). Sludge from secondary treatment processes are land-applied as biosolids, supplying CECs which may leach or run off into nearby bodies of water. Pharmaceuticals used in animal feeding operations may be released

to the environment in animal wastes via direct discharge of aquaculture products (i.e., antibiotics), the excretion of substances in animal urine and feces of livestock animals, and the washoff of topical treatments from livestock animals (Boxall et al. 2003).

EDCs discharged at WWTPs are one group of CECs with potentially widespread environmental effects (Folmar et al. 1996; Folmar et al. 2001; Jobling et al. 1998; Woodling et al. 2006). Although particular concern has been expressed about the anthropogenic EDCs, there are also natural estrogens (estradiol and its metabolites estriol and estrone) entering the aquatic environment through wastewater discharge and excretion from domestic animals. Furthermore, little is known about the environmental occurrence, fate and, transport for any of these compounds after they enter aquatic ecosystems. Many of the man-made compounds have been in use for a long time, and there is concern about pharmacologically active ingredients and personal care products that are designed to stimulate a physiological response in humans, plants, and animals (Daughton and Ternes 1999).

Frequent detection of compounds by itself does not constitute a need for ALC. Rather, criteria development for CECs needs to focus efforts on chemicals that demonstrate a reasonable potential to adversely affect aquatic life. Of CECs now known to be found in some surface waters of the U.S., EDCs have received the most attention because field studies from around the world have demonstrated that very low concentrations of some of these compounds can significantly impact natural populations of aquatic vertebrates. For example, observational field studies (Jobling et al. 1998) have shown a high occurrence of intersex (the presence of both male and female characteristics) in wild populations of a fish known as roach (*Rutilus rutilus*) in rivers in the United Kingdom that are downstream from WWTPs. Similar results have recently been reported for white sucker (*Catastomus commersoni*) in northern Colorado, U.S.A (Woodling et al. 2006). In a multiyear study by Kidd et al. (2007), the authors showed that environmentally relevant concentrations of ethynylestradiol, EE2, caused reproductive failure and near collapse of a natural fathead minnow population in an experimental lake, and also had deleterious effects on the reproductive biology of the pearl dace. These direct effects resulting in loss of forage fish have led to cascading effects on the lake trout population due to lack of prey (Kidd, personal communication). Researchers from the U.S. Geological Survey (USGS) have observed intersex and testis-ova (the presence of eggs in the testis) in bass species collected from the Potomac River and its tributaries in West Virginia, Maryland, and Washington DC, and also quantified EDCs in their blood (Blazer et al. 2007; Chambers and Leiker 2006). The occurrence of intersex fish in the Potomac River, as well as documented occurrence of this and related effects in other waters of the US and internationally, prompted Congressional hearings that were held in October 2006 to inquire about the “State of the Science on EDCs in the Environment,” as well as EPA activities associated with EDCs.

1.3 Purpose and Organization of This White Paper

The purpose of this white paper is to provide general guidance on how criteria development for CECs could be facilitated through a supplemental interpretation of the *Guidelines*, with particular attention to PPCPs with an EDC mode of action (MOA). Section 2 of this part (Part I) of the white paper describes the *Guidelines* procedures and identifies several areas in which these

procedures could be modified to address potential limitations for deriving criteria for CECs. Section 3 expands upon the areas of concern with respect to specific toxicological characteristics of some CECs. Section 4 summarizes these concerns and provides recommendations that could aid in the development of criteria for CECs in a resource efficient manner that takes best advantage of existing knowledge. Part II of this white paper further describes these concerns and recommendations using data for the synthetic pharmaceutical estrogen EE2.

2.0 CURRENT ALC METHODOLOGY

The *Guidelines* specify various data and procedural recommendations for criteria derivation, and also define general risk management goals for criteria, which are to provide a high level of protection for aquatic communities and for important species in these communities. ALC are defined to consist of two concentrations – the Criterion Maximum Concentration (CMC), intended to protect against severe acute effects, and the Criterion Continuous Concentration (CCC), intended to protect against longer term effects on survival, growth, and reproduction. The CMC is used in criteria to limit peak exposures by requiring that 1-hour averages of exposure concentrations not exceed the CMC more often than once in three years on average. The CCC is used in criteria to limit more prolonged exposures by requiring that 4-day averages of exposure concentrations not exceed the CCC more often than once in three years on average.

The CMC and CCC are usually derived from laboratory toxicity test results using specific standard procedures described in the *Guidelines*, but the *Guidelines* also have general provisions for deviating from these procedures as warranted by available information. The following text will first give an overview of the data requirements and calculations in the standard procedures, and then discuss how these procedures might vary under the umbrella of the *Guidelines*.

2.1 Standard ALC Derivation Procedures

The CMC is determined based on available Acute Values (AVs) – median lethal concentrations (LC_{50}s) or median effect (for a severe acute effect such as immobilization) concentrations (EC_{50}s) from aquatic animal acute toxicity tests (48- to 96-hours long) meeting certain data quality requirements. To compute a CMC, the *Guidelines* require that acceptable AVs be available for at least eight genera with a specified taxonomic diversity, in order to address a wide variety of the organisms constituting an aquatic animal community. These minimum data requirements include three vertebrates (a salmonid fish, a fish from a family other than salmonidae, and a species from a third chordate family) and five invertebrates (a planktonic crustacean, a benthic crustacean, an insect, a species from a phylum other than Chordata or Arthropoda, and a species from another order of insect or a fourth phylum).

For each genus, a Genus Mean Acute Value (GMAV) is calculated by first taking the geometric mean of the available AVs within each species (Species Mean Acute Value, SMAV) and then the geometric mean of the SMAVs within the genus. The fifth percentile of the set of GMAVs so obtained is calculated based on a specified estimation method, and designated the Final Acute Value (FAV). The FAV is then lowered to the SMAV for an important, sensitive species if appropriate. The CMC is set equal to half of the FAV to represent a low level of effect for the fifth percentile genus, rather than 50% effect.

The CCC is generally determined based on available Chronic Values (CVs), which are either (a) the geometric mean of the highest no-observed-effect concentration (NOEC) and lowest

observed effect concentration (LOEC) for effects on survival, growth, or reproduction in aquatic animal chronic tests or (b) in some recent criteria (e.g., ammonia), the EC_{20} in such tests based on concentration-effect regression analyses. Chronic tests for invertebrate species are required to include the entire life-cycle, but for fish partial life-cycle and early life-stage (ELS) testing protocols are accepted, the latter not including reproductive endpoints and not used if life-cycle or partial life-cycle tests are available and show more sensitive adverse effects.

If CVs are available for at least eight genera with the required taxonomic diversity, the CCC is set to the fifth percentile of Genus Mean Chronic Values (GMCVs), by the same procedure used to derive an FAV from GMAVs. Otherwise, the CCC is set to the FAV divided by a Final Acute Chronic Ratio" (FACR) that is based on acute to chronic ratios (ACRs – the ratio of the AV to the CV from parallel acute and chronic tests) for at least three species with a specified taxonomic diversity. The CCC can also be based on plant toxicity data if aquatic plants are more sensitive than aquatic animals, or on other data as deemed scientifically justified.

Further details on test requirements and calculation methods for the CMC and CCC are specified in the *Guidelines*, including deriving criteria that are a function of water quality characteristics.

2.2 Alternatives for ALC Derivation

The procedures described above enable broad application to toxic chemicals generally, and are only constrained by specific data requirements for quality and minimum taxonomic representation. Since they are not restricted with respect to specific types of chemicals, there is no reason to suppose that the standard data requirements and procedures specified by the *Guidelines* are any more or less applicable to CECs than to the chemicals for which criteria have already been developed. The *Guidelines* anticipated that rote application of the basic procedures may not yield the most appropriate criteria; consequently, the *Guidelines* provide flexibility when appropriate for deviation from the normal procedures regardless of the type of chemical, as indicated by the following provisions (hereafter referred to as the "Good Science" clauses:

> *"These National Guidelines should be modified whenever sound scientific evidence indicates that a national criterion produced using these Guidelines would probably be substantially overprotective or underprotective of aquatic organisms and their uses on a national basis."* (p. 18).
>
> *"On the basis of all available pertinent laboratory and field information, determine if the criterion is consistent with sound scientific evidence. If it is not, another criterion, either higher or lower, should be derived using appropriate modifications of these Guidelines."* (p. 57).

In addition, although the standard procedures in the *Guidelines* for deriving a CMC and CCC use only toxicity tests meeting certain requirements, the *Guidelines* also mandate the collation and examination of other data that might show effects that should be considered in criteria derivation:

> *"Pertinent information that could not be used in earlier sections might be available concerning adverse effects on aquatic organisms and their uses. The most important of these are data on ... any other adverse effect that has been shown to be biologically important. Especially important are data for species for which no other data are available. ... Such data might affect a criterion if the data were obtained with an important species, the test concentrations were measured, and the endpoint was biologically important."* (p. 54).

While alternatives are allowed when a specific situation dictates, the *Guidelines* still require that any changes in the procedures are consistent with the level of protection represented by the standard procedures:

> *"Derivation of numerical national water quality criteria for aquatic organisms and their uses is a complex process and requires knowledge in many areas of aquatic toxicology; any deviation from these Guidelines should be carefully considered to ensure that it is consistent with other parts of these Guidelines."* (p. iv).

Therefore, for the development of criteria for any chemical, the general strategy should be to start with the standard *Guidelines* procedures and then to adapt those procedures as warranted by available information on the effects of the chemical. This strategy applies to CECs as well, although certain considerations might more consistently be important for CECs. Specific attributes of CECs that might affect criteria derivations are considered in Section 3 of this paper, but several issues are introduced here that are of general concern.

Are data on acute toxicity needed for risk assessments?

Some chemicals are not acutely toxic even at concentrations so high that they could not possibly occur in the environment (e.g., at the chemical solubility, or exceeding exposures possible based on known chemical production and discharges). The acute lethality of some classes of chemicals might be measurable, but would occur at environmental concentrations so much higher than those affecting reproduction, growth, or chronic survival that, in practice, environmental exposures will always be far below acutely lethal levels if those exposures are managed to limit chronic effects. Therefore, derivation of the CMC might be unnecessary or impossible. Thus, if the existing data indicate that it is reasonably certain that acute toxicity would not occur at environmentally relevant concentrations, conducting additional acute tests is likely to be unwarranted.

Even if a CMC is not needed, another use of acute toxicity data is for developing "acute to chronic ratios" (ACRs) that are used with the FAV to calculate the CCC (see pages 40-42 in the *Guidelines*) , so that dropping acute testing requirements must consider this consequence as well. However, if acute effect concentrations are extremely high compared to chronic effect concentrations (large ACRs), whether the ACR approach should be even used warrants some consideration. Large ACRs are not, *per se*, less accurate than low ACRs, provided acute and chronic effect concentrations are well defined and the issue is simply extrapolating from acute to chronic toxicity within a species. However, for criteria calculations, the FACR needs to be a ratio that extrapolates from the fifth percentile of the acute effect concentration distribution to the fifth percentile of the chronic effect distribution. This requires appropriately combining ACR

information across species, the accuracy of which might be affected by large ACRs even if the accuracy of the individual ACRs is not. Therefore, in addition to not needing a CMC, extreme acute tolerance might also warrant direct calculation of the CCC rather than using the ACR approach, and thus eliminate the need for fulfilling all of the minimum acute toxicity test requirements as specified by the *Guidelines*.

How should data requirements for tolerant taxa be addressed?
The fifth percentile calculation methods for the CMC (as well as the CCC if the eight minimum data requirements noted above are met) require actual GMAV (or GMCV) values only for the four most sensitive genera. For more tolerant genera, it is only necessary to know that these toxicity values are greater than those of the four sensitive species. Therefore, toxicity test results that report "greater than" effect concentrations are acceptable for the tolerant taxa, and in fact are used in various criteria already.

If chronic tests have not already been done on some taxa needed for the minimum data requirements, but which are known to be tolerant, testing resources might be wasted by generating numbers that will not affect results. If methods such as inter-chemical or inter-species extrapolation methods, or assays (e.g., *in vitro* tests, biomarkers) that have been related to apical effects such as reductions in growth, survival, or reproduction can demonstrate these taxa to be insensitive compared to other taxa, actual chronic tests on these taxa may not be needed. In other words, can minimum data requirements for tolerant taxa be satisfied by some type of estimation rather than by an actual test result?

However, adding estimated data can become a rather open-ended process. Therefore, consideration must be given to how many estimated values should be allowed, relative to measured values, to produce an appropriate distribution of taxa in the data set used for criteria derivation.

Should fish chronic tests be required to address reproduction?
For chemicals (e.g., environmental estrogens) for which reproductive toxicity is of most concern, the allowance in the *Guidelines* for using ELS tests might need reconsideration. The *Guidelines* already give priority to life-cycle and partial life-cycle tests when they are available and show greater sensitivity than ELS tests. However, other information (from other species, similar chemicals, knowledge of the MOA) regarding latent or multigenerational reproductive effects may demonstrate the importance of sexual development and reproduction, so as to establish a basis for not considering ELS test results (or even partial life-cycle tests), but rather requiring life-cycle tests for fish.

What endpoints can serve as surrogates for traditional chronic endpoints?
Although chronic criteria are and will continue to be based on effects on reproduction, growth, and survival, another issue is whether only toxicity data directly addressing these endpoints is acceptable. Is there additional information (e.g., sub-organismal biomarkers, behavioral data) that can be used in criteria derivation because they are adequately correlated to reproduction, growth, and survival? Use of such data would be consistent with the *Guidelines* requirements to examine all pertinent data and make modifications to the criteria derivation procedures that are consistent with sound scientific evidence

2.3 Precedent for Deviating from Basic ALC Derivation Procedures

The recent ALC document for tributyltin (TBT) provides a good example of some of the types of procedural criteria modifications discussed above. TBT is a highly toxic biocide that has been used extensively in anti-fouling paint to protect the hulls of large ocean-going ships. It is deemed a problem in the aquatic environment because it is extremely toxic to non-target organisms, and has been linked to imposex (the superimposition of male anatomical characteristics on females) and to immuno-supression in snails and bivalves (U.S. EPA 2003). The concentrations reported to cause imposex in the laboratory are lower (range: 0.0093 to 0.0334 µg/L) than the FCV (0.0658 µg/L) calculated using the standard ALC derivation procedures (U.S. EPA 2003). The low effect concentrations established for female gastropods in the laboratory were subsequently corroborated in field studies. The CCC was lowered (to 0.0074 µg/L) based on the judgment that these effects were relevant for the risks of TBT to gastropod reproduction.

3.0 CHARACTERISTICS OF CECS AND THEIR INFLUENCE ON ALC DEVELOPMENT

As described in Section 1.0, chemicals become labeled as CECs for a variety of reasons, many of which have relatively little to do with their toxicological characteristics. Consequently, the Guidelines cannot be interpreted or modified in one particular way that would be universally appropriate for all CECs. However, some characteristics may be shared by various CECs, such that discussing the implications of these characteristics in the context of deriving water quality criteria is worthwhile. The expected outcome is additional guidance addressing key issues that may arise and how best to accommodate these issues in deriving criteria.

Much of the technical discussion that follows is centered on EDCs and, even more specifically, around chemicals that interact with the hypothalamic/pituitary/gonadal (HPG) axis. Endocrine function controlled via the HPG axis involves hormones broadly known as estrogens ("female" hormones such as estradiol) and androgens ("male" hormones such as testosterone), along with the body tissues and biochemical machinery with which they interact. Effects on this part of the endocrine system of various aquatic species have been documented in the literature, and publicized in the media, making toxicological disruption of this mechanism a good choice for discussing CECs in the context of the *Guidelines*. However, these types of substances are only a subset of EDCs, and an even smaller subset of CECs as a whole. While much of the discussion that follows uses HPG-active chemicals as a point of reference, the concepts presented may be useful in the derivation of ALC for many other chemicals as well. It is the principles more than the specifics that are important in considering the content of this report.

3.1 Characteristics of HPG-Active EDCs

While estrogenic and androgenic hormones are a core component of the HPG axis, this system also includes a much larger group of tissues and biochemical machinery within the body which, in vertebrates, govern sexual development, maturation, and reproduction. Commensurate with this complexity, there are many places within the system that environmental chemicals may act to modify the normal function of the HPG-axis. Thinking simply of "estrogenicity" or "androgenicity" as toxicological modes of action is still too broad – these categorical classes are more the outward "symptoms" of disruption in the HPG axis than they are unique modes of toxicological action. For example, the synthetic steroids EE2 and trenbolone bind to (and act as agonists of) vertebrate estrogen and androgen receptors, respectively, with similar or greater affinity than the natural endogenous hormones, estradiol and testosterone. In contrast, a variety of other medicinal pharmaceuticals are specifically designed to do the opposite, to be antagonists of these same receptors. As examples, tamoxifen (breast cancer treatment) and flutamide (prostate cancer treatment) bind quite specifically to vertebrate estrogen and androgen receptors, respectively, thereby blocking the activity of endogenous steroid hormones. But disruptors of the HPG axis are not limited to chemicals that bind directly to estrogen or androgen receptors; they also include chemicals that interact elsewhere in the overall biochemical pathway. As an example, there are chemicals that exert their activity through interactions with CYP (cytochrome

P450) enzymes involved in steroid production. The pharmaceutical chemical fadrozole acts to inhibit CYP19 aromatase, the enzyme that converts estradiol to testosterone. A number of conazole fungicides act as competitive inhibitors of several CYPs further up the steroid synthesis pathway (Ankley et al. 2005).

Unlike many other chemicals that have either non-specific (e.g., narcotics) or more generalized reactive modes of action (e.g., electrophilic chemicals interacting with nucleic acids and proteins), HPG-active compounds tend to have very specific interactions with particular molecular targets within the biochemical pathway. There are a number of consequences arising from this specificity. One important consequence of target specificity is potency. Many pharmaceuticals are designed to be highly specific, and thus are extremely potent. For example, EE2 and trenbolone affect reproduction and development in fish at water concentrations in the very low ng/L (part-per-trillion) range (e.g., Ankley et al. 2003; Länge et al. 2001), well below effect concentrations for most chemicals for which current ALC have been derived. These very low biologically-active concentrations present substantial challenges for analytical determinations associated either with lab-based effects testing or field monitoring of *in situ* exposures. In the case of EE2 and/or trenbolone, effects observed in fish are at concentration levels below the methodological limit of detection for most laboratories even in laboratory test water, and even more so ambient water and effluents.

Such high potency can influence how one would approach criteria derivation when the chemicals exert minimal acute toxicity, but cause mostly long-term, sub-lethal effects. Trenbolone and EE2 illustrate this situation quite well. Like most pharmaceuticals (some exceptions being chemotherapy and anti-parasitical agents), these chemicals are designed to "adjust" the biochemistry of the body without causing acute toxicity or other significantly adverse side effects. As a consequence, these types of pharmaceuticals tend to have low toxicity in short-term lethality assays (Webb 2001). In the context of criteria development, this has implications for the use of ACRs. Most conventional toxic pollutants with EPA ALC have ACRs of 10 or less (Cunningham et al. 2006; Host et al. 1995). In contrast, ACRs for EE2 and 17β-trenbolone in fish have been shown to range from 1,000 to greater than 300,000 (Ankley et al. 2005). Again, this is a result of relatively low acute toxicity and high chronic potency. Importantly, limited data for other MOA classes of pharmaceuticals suggest that this phenomenon is not restricted to endocrine-active substances. For example, Huggett et al. (2002) reported an ACR in fish of about 50,000 for propanolol, a β-blocker. As discussed in Section 2, this large difference in acute and chronic potency may both make CMC values moot in the environmental management of these chemicals, and introduce uncertainty in the extrapolation between acute and chronic effects in the derivation of a CCC.

The specificity of the molecular target also can greatly affect those taxa likely to be sensitive to the chemical MOA of concern. While some biological pathways (e.g., energy metabolism) tend to be highly conserved across all organisms, others can be quite specific to certain phylogenetic groups. Although the control of reproductive function through the HPG axis is highly conserved across vertebrate classes, lower taxonomic groups such as invertebrates have different endocrine system structure that function differently. For example, Segner et al. (2003) tested several estrogenic chemicals, including EE2, in a variety of partial and full life-cycle tests with a model fish (zebrafish) and several aquatic invertebrate species. They found that the fish was by far most

sensitive to the effects of the estrogenic chemicals, and was the only species that responded to EE2 at environmentally-relevant concentrations. As a result, it is likely that chronic toxicity data for fish would be the most influential in setting the criterion for EE2, and correspondingly unlikely that toxicological data for invertebrate species would have much impact. Plants do not have comparable endocrine system structure or function, and would not be expected to be sensitive to these types of compounds, but there is research that indicates that algal species may be a uniquely sensitive taxa for the assessment of other types of CECs such as antibacterial products like triclosan (Orvos et al. 2002; Wilson et al. 2003).

Specificity in MOA can also affect how or if effects are expressed within a toxicity test, even in potentially sensitive species. In the case of chemicals that affect endocrine function, there are distinct "windows" when animals are likely to be sensitive and/or exhibit adverse outcomes. For example, a popular amphibian early developmental assay-FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) would be inadequate for detecting thyroid-active toxicants because the period of exposure and observation occurs early in development before the thyroid axis is functional in *Xenopus*. In the case of HPG-active toxicants, there are two windows of sensitivity during an animal's life: during sexual differentiation in developing organisms (when "organizational" alterations occur), and during active reproduction in adults (when "activational" responses can be manifested; Ankley and Johnson 2004). As a result, it is critical that testing for HPG-active EDCs occur during periods when the system is vulnerable to disruption. It is equally critical that toxicity tests include observations during the periods when effects are expressed. Some of the changes caused during sensitive early developmental windows may not be expressed until later in life. For example, the ELS toxicity test protocol commonly used in criteria development to estimate the chronic sensitivity of fish contains the early life stages that could be sensitive to disruption of sexual development, but it does not extend through to maturation, and would therefore be insensitive for detecting disruption of sexual development.

3.2 Implications for ALC Development

As is clear from the text above, some characteristics of HPG-active chemicals (and many other CECs) create the need to carefully interpret the intent of the *Guidelines*, not just the routine derivation process. As indicated in Section 2.0 there is nothing about CECs that invalidates the principles embodied in the *Guidelines*; however, the *Guidelines* were written before many of the issues discussed in Section 3.1 were known, so they do not necessarily contain prescriptive guidance for some of the nuances created by some CECs. The following paragraphs discuss the implications of these issues for criteria development, following the four general topic areas outlined in Section 2.0:

- The need for and relevance of acute toxicity data and a CMC;
- Defining minimum data requirements in terms of taxonomic coverage;
- Defining appropriate chronic toxicity data; and
- Selecting effect endpoints upon which to base criteria

3.2.1 The Need For and Relevance of Acute Toxicity Data and a CMC

As described in Section 2.2, there are two primary uses for acute toxicity data under the *Guidelines*: 1) the derivation of the CMC; and 2) establishment of the CCC when the FAV/FACR method is used. As a practical matter, if the CMC is more than 96-fold higher than the CCC, then it will always be the CCC that is more limiting. This is because in the standard formulation of criteria, the CMC has a one-hour averaging period and the CCC a 96-hour averaging period; thus, if the difference between them is more than 96-fold, it is mathematically impossible to exceed the CMC without also exceeding the CCC. A minor exception to this issue occurs when ALC are implemented in an NPDES permit such that the CMC is applied to whole effluent while the CCC is applied after mixing, and the available in-stream dilution is large. However, these exceptions are rare, and even the 96-fold difference discussed here pales in comparison to the factors of 1,000 to 300,000-fold discussed previously in regard to EE2 and trenbolone. In cases where such extreme differences between acute and chronic toxicity thresholds exist, establishing ALC as having only a CCC seems a reasonable approach.

While it is easy to see why a CMC would not be necessary when you have sufficient acute test data to show that the CMC would be dramatically higher than the CCC, this begs the question of how much data are needed to decide that this is the case. This decision should occur during the "problem formulation" step in the risk assessment for a specific chemical/class, and should be guided by the following types of information:

- the amount and phylogenetic spread of acute toxicity data that are available;
- toxicity data from short-term exposures that do not meet the strict definitions in the *Guidelines* of acute toxicity data acceptable for criteria derivation, but from which information on responses to acute exposures can be inferred;
- short-term effect data garnered from longer-term exposures;
- information from closely related chemicals that are thought to have the same MOA, and have more robust acute data sets; and
- knowledge of the degree of phylogenetic distribution of the toxicity pathway of concern.

A complicating issue resulting from a "moot" CMC is that data availability for acute effects will likely be limited. As such, having less than the required acute MDRs may preclude the ability to derive a CCC using the FAV/FACR approach typically used in the *Guidelines*. For chemicals with highly specific modes of action and large ACRs (such as many EDCs), it is very likely that the mechanisms for acute and chronic toxicity differ, since biological activity resulting in chronic effects is designed into the product and not a secondary consequence - such as many of the historical contaminants for which EPA has developed criteria. Also, sensitivity of different taxa classes to acute and/or chronic toxicity varies widely due to presence (or absence) and structure and function of phylogenetically-conserved systems. Both of these issues would introduce considerable uncertainty into the availability and interpretation of ACRs, and probably make it inadvisable to use the FAV/FACR approach anyway. The *Guidelines* discuss the inadvisability of using the ACR approach when ACRs vary by more than a factor of 10 without a clear relationship to taxonomy or acute sensitivity (page 41 of the *Guidelines*). A more advisable approach would generally be to develop a CCC directly from a sufficiently robust set of chronic data, using the procedures outlined in the *Guidelines* or an appropriate modification thereof.

3.2.2 Defining Minimum Data Requirements in Terms of Taxonomic Coverage

To develop a CCC directly from chronic toxicity data (rather than via FAV/FACR), the *Guidelines* require that acceptable chronic toxicity data be available from at least eight families with a taxonomic distribution fulfilling the requirements specified in the *Guidelines* (referred to as the "minimum data requirements" or "MDRs"). Having a blanket requirement for meeting the eight MDRs was included to insure a minimum level of "certainty" that the *Guidelines* will be protective of the broad phylogenetic distribution of organisms found in aquatic systems. Including this phylogenetic spread also enables criteria to be developed for chemicals for which the toxicological MOA is not known. Instead of "knowing" what organisms are most likely to be sensitive to a particular chemical, requiring a broad spread of empirical toxicity data makes it likely that whatever taxa may be sensitive to a chemical, they will be represented to some degree in the toxicity data set.

In the case of EDCs, PPCPs, and certain other chemical classes, we may have a reasonable understanding of the toxicological MOA for the chemical, and from that knowledge we may be able to infer what taxa are most likely to be sensitive to a particular chemical (Ankley et al. 2007; Williams 2005). As discussed in Section 2.2, the procedure used in the *Guidelines* for estimating the 5^{th} percentile of a toxicity distribution is dependent on only the four lowest values; for this reason exact values for insensitive genera are not necessary, as long as there are sufficient data to infer that their sensitivity is lower than the four most sensitive genera.

So how does one determine that a particular taxon is insensitive? The general structure of the *Guidelines* presumes that sensitivity is determined by conducting an acceptable toxicity test with that taxon. However, one can infer that the actual need is only to have sufficient information to conclude that the taxon is insensitive; if that determination can be confidently made based on other information, the information need may be met even if an actual toxicity test with that particular organism and chemical has not been conducted. This does not change the intent of the *Guidelines*. It only acknowledges the possibility that there is more than one way to meet the information requirement.

Using the example of EE2, there is both physiological understanding and some empirical toxicity data to support the belief that vertebrates will be far more sensitive to EE2 toxicity than will invertebrates (see Part II of this white paper and Segner et al. 2003). As such, it would seem inappropriate to invest resources in testing a wide range of invertebrate taxa classes for sensitivity when all existing data indicate that the data would not affect the final criterion, which would be driven by sensitivity of vertebrates. In this case, it makes sense to argue that certain of the eight MDRs might be declared met not through direct testing, but through toxicological understanding of the chemical's MOA and the physiology of those other taxa or existing toxicity data that establishes sensitivity differences among taxa.

While this logic is clear, one must be careful in presuming that the primary MOA demonstrated by organisms with the target physiology is the only toxic MOA for the chemical. Particularly given the phylogenetic diversity of organisms, it is always possible that a chemical that behaves

with one MOA in one class of organisms may exert toxicity through a different mechanism in a different phylogenetic group. There are precedents for this scenario (Ankley et al. 2007). For example, exposure to the non-steroidal anti-inflammatory drug diclofenac via consumption of dead livestock has greatly diminished some populations of vultures in several East Asian countries. Diclofenac kills the birds through renal failure, which is only a relatively minor side-effect of the drug in mammals. While the mechanism of renal toxicity in vultures is likely molecularly related to the mechanism of therapeutic action in man, i.e., both seem to occur due to inhibition of prostaglandin synthesis (Meteyer et al. 2005), the inhibition of similar molecular components appears to be manifested as dramatically different whole organism endpoints. The key is in achieving a reasonable balance between expending resources on collecting data most likely to influence the criterion, while maintaining some kind of backstop against initially unexpected toxicity in other organisms.

One possibility for enhancing confidence regarding phylogenetic sensitivity is in considering data for other, closely-related chemicals with the same MOA. While the *Guidelines* focus analysis on toxicity data for the specific chemical in question, an understanding of toxicological MOA can also lead to an understanding of how other chemicals might act to exploit the same biological system in the same way. For example, one might reasonably infer that the relative species sensitivity to EE2 is likely similar to 17-ß estradiol (E2), the natural hormone which EE2 mimics. If, for example, there were a taxon which had been tested and found insensitive to E2, but had not been tested with EE2, it seems a reasonable assumption that that taxon would also be insensitive to EE2.

The possibility of fulfilling certain information requirements using data other than from direct toxicity testing does raise some other interpretation challenges, in particular the definition of the sample size for determining the 5^{th} percentile of the genera sensitivity distribution. For example, if one has reason to believe that all crustaceans would be insensitive to a chemical, how many genera does that assertion represent in the calculation of the genera sensitivity distribution? While this is a real issue that will have to be addressed, we believe the problem is tractable and the details of the resolution are left to later work.

Because of the risk that our mechanistic understanding of a chemical may be incomplete, it seems unlikely that one could justify completely bypassing several MDRs solely on theoretical arguments (e.g., developing a criterion for a testosterone mimic based only on chronic toxicity data for vertebrates, with no invertebrate data at all). At the same time, prudent application of other data types to fulfill certain information requirements for criteria derivation does seem appropriate. Given the tremendous variation in understanding and availability of data likely to exist for different CECs, it is presumed that at least initial application of this approach will have to be justified on a chemical-by-chemical basis using appropriate scientific judgment. However, lines of evidence that might be applicable to this determination include:

- an in-depth understanding of the toxicological (or, in the case of drugs, therapeutic) MOA;
- information on the basic physiology of other taxa in relation to the MOA;
- toxicity data from chronic exposures or other relevant experiments that do not meet the strict definitions of acceptable chronic data given in the *Guidelines*, but from which

information on relative taxon sensitivity can be inferred; and

- information from closely related chemicals that are thought to have the same MOA and have more robust acute or chronic data sets.

A separate, but related issue arises in respect to data from species not resident to North America. The *Guidelines* specify the use of toxicity data only from species resident to North America. However, particularly in regard to the study of EDCs, some fish species not resident to the U.S. have been advanced as experimental models for the evaluation of the chronic effects of EDCs to fish. Two clear examples at the time of this report are the zebrafish (*Brachydanio rerio*) and the Japanese medaka (*Oryzias latipes*), for which equivalency of EDC test data (with the fathead minnow) has been proposed through international groups such as the Organization for Economic Cooperation and Development (OECD; Ankley and Johnson 2004). These species have a rich toxicological database, and we know of no reason to believe that their sensitivities would be expected to be substantively different from sensitivities of at least some fish resident to the U.S. In keeping with international harmonization, we suggest that toxicity data from species with recognized international equivalency be included in criteria derivation with the full weight given to data from resident species.

3.2.3 Defining Appropriate Chronic Toxicity Data

The *Guidelines* state that acceptable chronic tests for criteria derivation are full life-cycle exposures (F_0 egg to F_1 offspring) for both vertebrates and invertebrates, as well as partial life-cycle (adult to juvenile) and early life-stage (ELS; egg to juvenile) tests for fish. The acceptance of ELS tests in particular as acceptable chronic tests is predicated on the work of McKim et al. (1978) and other evidence that the toxicity thresholds obtained from ELS tests are generally within a factor of two of the thresholds from life-cycle chronic tests.

While this general approach has been applied with apparent success for many chemicals, the *Guidelines* intimate concerns with the approach, noting that for some chemicals, ELS tests might not be good predictors of chronic toxicity, which would violate the principle underlying the use of ELS tests as chronic data (page 39 in the *Guidelines*). As noted previously, toxicological data for chemicals like EE2 show that certain chemicals may have potent effects on life processes that lie outside the exposure period represented by ELS tests (e.g., pronounced effects on reproduction), or on life processes for which the expression of effects does not occur until after the ELS period (e.g., embryo or larval exposure resulting in effects on sexual development and maturation in adult fish; see Section 3.1). It is clear from these examples that there are chemicals for which ELS tests should not be used as surrogates for full life-cycle exposures. In fact, chemicals that affect sexual differentiation may not be adequately assessed even with partial life-cycle exposures, since these protocols do not generally include observation of sexual development/maturation in fish exposed during early development.

While the “Good Science” clause and other text in the *Guidelines* would not support reliance on ELS tests as chronic data for chemicals known to have specific effects on other life processes, such as sexual development or reproduction, the *Guidelines* would allow the development of a criterion using only ELS data for fish if there were not any specific data to indicate that this approach would be inappropriate. This is akin to an “innocent until proven guilty” approach.

However, we believe experiences with chemicals like EDCs make clear the need to move from the previous approach to one of "guilty until proven innocent." In other words, it is probably wiser to require that the chronic toxicity data for fish include exposure and observation over a full life-cycle unless there is an affirmative reason to believe that it is not necessary (note: this issue is equally relevant to invertebrates species, but the ELS tests discussed in the *Guidelines* are focused explicitly on fish; invertebrate tests would already be required to be life-cycle). In keeping with this shift in emphasis, we believe the requirements for chronic toxicity data in the *Guidelines* should be tightened by adding the further requirements that either:

1) Full life-cycle data be available for at least one fish species; or

2) There is a body of experimental information indicating that life processes outside the ELS or partial life-cycle exposure/observation windows would not be important to capturing the important toxicological effects of the chemical.

At first glance, #2 may seem like requiring the proof of a negative, in that one would have to actually conduct the life-cycle test required by #1 in order to show that #2 is true. However, we believe there may be circumstances in which there may be data that speak to the sensitivity of different life stages that come from studies that, while scientifically valid, for some reason do not meet all the requirements of a valid life-cycle test as defined in the *Guidelines*. For example, there may be data for a life-cycle test with a non-resident species that includes the relevant life processes but does not qualify as an acceptable chronic test for the derivation of criteria because it is non-resident. Alternatively, there may be data from experiments that violate other requirements of acceptable toxicity data under the *Guidelines*, but still provide insight into sensitive exposure periods or life processes. Even though CVs from such data may not be used directly to calculate a chronic criterion, it seems reasonable to use such data to evaluate the question of where in the life-cycle there are important windows of exposure and/or effect and how that constrains the adequacy of ELS tests to represent chronic toxicity. In other cases, there may be sufficient information from other types of research to demonstrate to a reasonable level of certainty that a chemical's toxicological mechanism(s) would not preclude the use of ELS tests as indicators of chronic toxicity.

Where life-cycle toxicity data are available, the results of those experiments should be carefully examined to determine the likelihood that important windows of exposure or effect lie outside ELS test protocols. Obviously, if there is meaningful potential for effects outside the ELS exposure period, ELS tests should not be used as surrogates for more involved chronic tests. It may also be that the knowledge of toxicological mechanism for a particular chemical may be sufficient that meaningful chronic toxicity data could be developed from exposures that have a structure different from the life-cycle, partial life-cycle, and ELS protocols defined explicitly in the *Guidelines*. While defining such alternate protocols is beyond the scope of this document, we recognize the potential for such a situation and leave it to appropriate implementation of the "Good Science" clause to allow for inclusion of such alternative exposure protocols as surrogates for chronic toxicity data, most likely in addition to, rather than instead of, data from life-cycle toxicity tests.

At the other end of the spectrum lie toxicity tests that extend beyond the definition of a full life-

cycle test, often referred to as multi-generational tests. Because they encompass the full range of life processes as a life-cycle test, we feel that they should be included as acceptable chronic tests, assuming they meet all other requirements for test acceptability. Some studies have reported effects from EDC or other chemicals in which exposure to one generation creates effects in a later generation that were not observed in prior generations even at the same life stage (Nash et al. 2004). If substantial, such effects could create a situation where even full life-cycle toxicity tests might underestimate the chronic toxicity of a chemical and therefore produce criteria that are potentially under-protective. While we recognize the potential for this situation, at present we believe there is not sufficient reason to make multi-generational testing a requirement for criteria development, unless there is specific, compelling information that a criterion would be substantially under-protective if multi-generational effects were not rigorously considered.

3.2.4 Selecting Effect Endpoints Upon Which to Base ALC

The selection of endpoints appropriate to the derivation of ALC must be tied to the narrative intent of the overall *Guidelines*. The stated goal of ALC is to "protect aquatic organisms and their uses" (see *Water Quality Standards Handbook*; U.S. EPA 1994b). While the exact meaning of "protection" is not defined, there is considerable discussion in the *Guidelines* document that makes clear that protection does not mean the prevention of any measurable biological effect in any organism. Instead, there is discussion of endpoints that are "biologically important" and prevention of "unacceptable effects"; this implies that in the context of criteria there are effects that are "biologically unimportant" and/or levels of effect that are "acceptable." Related concepts include the idea that natural populations can withstand some magnitude/frequency of disturbance and still meet the intent of the *Guidelines*.

With "protection of aquatic organisms and their uses" as the assessment endpoint, a decision must be reached as to which biological responses (measurement endpoints in risk assessment parlance) are appropriate to address this goal. Survival, growth, and reproduction are processes that are generally accepted as being directly related to this goal, as these are all demographic parameters that directly affect population dynamics (although, the exact quantitative relationship is not always fully determined). However, there are many more biological responses that have been observed in response to toxicant exposure, both at the whole organism level (e.g., behavior) and at lower levels of biological organization (e.g., biochemical or histological changes). For many of these endpoints, their relationship to the assessment goal, "protection of aquatic organisms and their uses," is less clear. In this regard, we must consider an additional goal of the *Guidelines* – that criteria "provide a reasonable and adequate amount of protection, with only a small possibility of considerable overprotection or under-protection" (page 5 of the *Guidelines*). In keeping with this intent, it is important that criteria focus on endpoints that affect the assessment endpoint, but not create overprotection by preventing any measurable effect (or possibility of that effect). There must be a reasonable, affirmative connection between the measured response and the assessment endpoint.

The Agency's *Framework for Ecological Risk Assessment* (U.S. EPA 1992) identifies this problem:

> *In many cases, measurement endpoints at lower levels of biological organization may be more sensitive than those at higher levels. However, because of compensatory mechanisms and other factors, a change in a measurement endpoint at a lower organizational level (e.g., a biochemical alteration) may not necessarily be reflected in changes at a higher level (e.g., population effects).* (p. 14)

And later on:

> *Ideally, the stressor-response evaluation quantifies the relationship between the stressor and the assessment endpoint. When the assessment endpoint can be measured, this analysis is straightforward. When it cannot be measured, the relationship between the stressor and measurement endpoint is established first then additional extrapolations, analyses, and assumptions are used to predict or infer changes in the assessment endpoint.* (p. 23)

> *Measurement endpoints are related to assessment endpoints using the logical structure presented in the conceptual model. In some cases, quantitative methods and models are available, but often the relationship can be described only qualitatively. Because of the lack of standard methods for many of these analyses, professional judgment is an essential component of the evaluation. It is important to clearly explain the rationale for any analyses and assumptions.* (p. 23)

Existing criteria documents contain many types of data that were not used in the criteria derivation (the documents collate and review these data, but they are not used to actually define the criterion concentration) and it is useful to the discussion here to consider how such data have been interpreted. For example, the following text is derived from the most recent criteria document for ammonia (U.S. EPA 1999, see Appendix 5):

> *Endpoint indices of abnormalities such as reduced growth, impaired reproduction, reduced survival, and gross anatomical deformities are clinical expressions of altered structure and function that originate at the cellular level. Any lesion observed in the test organism is cause for concern and such lesions often provide useful insight into the potential adverse clinical and subclinical effects of such toxicants as ammonia. For purposes of protecting human health or welfare these subclinical manifestations often serve useful in establishing 'safe' exposure conditions for certain sensitive individuals within a population.*

> *With fish and other aquatic organisms the significance of the adverse effect can be used in the derivation of criteria only after demonstration of adverse effects at the population level, such as reduced survival, growth, or reproduction. Many of the data indicate that the concentrations of ammonia that have adverse effects on cells and tissues do not correspondingly cause adverse effects on survival, growth, or reproduction. No data are available that quantitatively and systematically link the effects that ammonia is reported to have on fish tissues with effects at the population level. This is not to say that the investigators who*

reported both tissue effects and population effects within the same research did not correlate the observed tissue lesions and cellular changes with effects on survival, growth, or reproduction, and ammonia concentrations. Many did, but they did not attempt to relate their observations to ammonia concentrations that would be safe for populations of fish under field conditions nor did they attempt to quantify (e.g., increase in respiratory diffusion distance associated with gill hyperplasia) the tissue damage and cellular changes (Lloyd 1980; Malins 1982). Additionally, for the purpose of deriving ambient water quality criteria, ammonia-induced lesions and cellular changes must be quantified and positively correlated with increasing exposures to ammonia.

In summary, the following have been reported:

1. Fish recover from some histopathological effects when placed in water that does not contain added ammonia.

2. Some histopathological effects are temporary during continuous exposure of fish to ammonia.

3. Some histopathological effects have occurred at concentrations of ammonia that did not adversely affect survival, growth, or reproduction during the same exposures.

Because of the lack of a clear connection between histopathological effects and effects on populations, histopathological endpoints are not used in the derivation of the new criterion, but the possibility of a connection should be the subject of further research.

As discussed in greater detail below, chemicals such as EDCs have been shown to produce a wide variety of measurable changes at many different levels of biological organization. The challenge is to select from among those the endpoints that have sufficiently clear connection to expected effects on populations of aquatic organisms.

3.2.4.1 Specific Examples of Measurable Changes at Different Levels of Biological Organization

The range of organismal endpoints that have been reported in the literature is vast, and varies to some degree on the organism and toxicant. With respect to only the HPG axis in vertebrates, this range of endpoints over and above direct measures of survival, growth, and reproduction includes:

- Biochemical measures (e.g., the female-specific yolk precursor protein vitellogenin; native hormones estradiol, testosterone, 11-ketotestosterone);
- Histopathological measures (e.g., proportion of spermatogonia, presence of testis-ova, oocyte atresia, Leydig cell hyperplasia/hypertrophy);

- Gross morphology (e.g., secondary sex characteristics: nuptial tubercles, coloration, ovipositors); and
- Behavioral measures (e.g., nest building, defense/aggression).

A comprehensive survey and evaluation of all such endpoints is far beyond the scope of this document. In lieu of that, this section presents in depth discussion of several individual measures relevant to the HPG axis, including their strengths and weaknesses as direct indicators of likely population level effects. The point of this discussion is simply to provide examples of the issues that must be considered in making a decision as to the biological importance and scientific defensibility for a specific endpoint, organism, or toxicant as it pertains to ALC derivation. These decisions will likely require case by case consideration; in certain circumstances, the suitability of a particular endpoint may vary across chemicals depending on how an individual chemical influences that endpoint.

One of the challenges that arises when incorporating alternative endpoints into criteria derivation is the need to not only conclude that the endpoint warrants consideration, but also establish some definition of what level of effect on that endpoint is unacceptable. While these links may not be completely quantitative, one would not want the definition of an unacceptable effect on one endpoint to be grossly disproportional to that considered unacceptable for another (i.e., if a 20% reduction in reproduction is considered unacceptable, what degree of estradiol (E2) suppression is equivalent to a 20% reduction in reproduction?).

In the text that follows, endpoints are categorized as being either “organizational” or “activational.” Organizational endpoints are those that are a result of changes to the normal growth and development of an organism, and are generally not reversible with cessation of exposure. Activational endpoints are those that occur in comparatively plastic tissues in response to exposure, but which may revert to their prior or normal condition with cessation of exposure.

Organizational Endpoint: Sex Reversal

Exposure of developing fish to endocrine-active materials during sensitive “windows” in early development can skew phenotypic sex dramatically toward either females (estrogenic chemicals) or males (androgenic chemicals). This response has been exploited by aquaculturists, who for many years have used potent natural or synthetic steroids to produce mono-sex stocks. The sensitivity of fish to this type of “sex reversal” is species-specific, and critical windows of exposure can vary markedly across species. The response can be manifested in several different ways, ranging from more or less completely sex-reversed animals (i.e., occurrence of gonads and secondary sex characteristics completely reflective of the opposite sex) to more subtle changes, such as the occurrence of intersex gonads (discussed further below). A significant challenge in assessing this condition-either in the lab or field-is knowledge of actual genetic sex of the fish. Since the molecular basis of sex determination in many fish is not known, reliable genetic markers of what sex an animal is programmed to be are not available for most test species (one notable exception here is the Japanese medaka, which is commonly used for endocrine testing in some parts of the world; Ankley and Johnson 2004). The net result of this is that the only way to practically monitor sex reversal in most fish species is indirectly, through analysis of sex ratios (generally based on phenotypic sex). This requires, of course, considerable background

knowledge concerning “normal” sex ratios for a species (or even strain) of fish. For some lab test species (e.g., fathead minnow), the normal sex ratio appears to be about 1:1, while for other commonly-tested small fish models (e.g., zebrafish), the ratio can be quite variable (Ankley and Johnson 2004). In field studies, collection of accurate sex ratio data also can be exceedingly difficult, depending on variables such as sampling gear and location and timing of collections.

Changes in the sex ratio of populations of fish, either in the lab or field, can be quite indicative of an endocrine-specific MOA, indicating exposure to estrogenic or androgenic chemicals (or even chemicals that block the actions of sex steroids). Significantly, from a risk assessment perspective, alterations in sex ratio could also have direct implications for spawning success and population viability. The degree to which sex ratios are critical in determining embryo production will vary based on reproductive strategies of the species of concern (e.g., broadcast versus paired spawners); however, from an evolutionary perspective, one would speculate that any departure from normal sex ratios for a species/population might be considered maladaptive.

Organizational Endpoint: Intersex

Exposure to certain classes of endocrine-active chemicals during critical windows in early development can produce intersex gonads (commonly termed testis-ova), a situation in which the gonads simultaneously contain both ovarian and testicular tissue. Different studies from around the world have shown an elevated occurrence of intersex fish downstream of municipal effluents containing natural and synthetic steroidal estrogens, including EE2 (WHO 2002). In fact, collection of intersex fish from the field has been one of the most visible manifestations of the effects of EDCs on fish/wildlife. For example, in a widely publicized USGS study, Blazer et al. (2007) recently reported that in the South Branch of the Potomac River and select nearby drainages, more than 80 percent of all the male smallmouth bass sampled had oocytes growing within their testicular tissue. Although histology is required to determine and quantify intersex, the techniques involved are relatively straight-forward. What is more challenging than measurement is interpretation of the results. For example, it appears that some degree of background intersex can occur, even in species held under carefully-controlled conditions (Grim et al. 2007). The degree of background intersex and sensitivity to chemically-induced intersex appear to be quite species-specific, requiring a thorough understanding of normal gonad differentiation and development in the species of concern.

Even in species for which background intersex is low, there is uncertainty as to the degree to which the condition could occur and not interfere with normal reproductive function. For example, in a field study in the UK, Jobling et al. (1998) noted a wide range of intersex in roach collected, even from the same site, with severity of the response ranging from occurrence of a few primary oocytes in otherwise normal testicular tissue to instances where there was a complete absence of sperm ducts in the males. Arguably, the former fish could produce viable sperm, while the latter certainly would not. So, although intersex is an intuitively reasonable endpoint upon which to base predictions of possible adverse effects of endocrine-active chemicals on reproductive success, determination of the relationship between severity of the condition and production of viable embryos is required to conduct this analysis.

Activational Endpoint: Behavior

Although not usually considered a biomarker in the traditional sense, behavior is an endpoint that historically has been seldom used for ecological risk assessment, including the derivation of ALC. There are several reasons for this: (1) the types of assays used to assess behavior can be quite labor-intensive, (2) many methods for assessing toxicant-induced behavior have some degree of subjectivity, (3) many behavioral changes (e.g., gill ventilation in fish) are relatively non-specific in that they do not necessarily reflect exposure to chemicals with a specific MOA, and (4) translation of behavioral changes into adverse effects on endpoints such as survival, growth and reproduction can be difficult. Nonetheless, virtually all environmental toxicologists recognize the potential for chemically-induced alterations in behavior to influence the health of individual animals and populations.

There are some compelling reasons to consider behavior as a potentially useful/important endpoint in assessing the ecological risk of certain classes of endocrine-active chemicals. First, estrogens and androgens are known to play relatively specific roles in a variety of reproductive behaviors in fish, including competition for mates, courtship and nest-holding/guarding. Alterations in any of these behaviors theoretically could affect reproductive success and, hence, population status. In recognition of this there have been several recent papers describing straight-forward, relatively quantitative assays for assessing the effects of endocrine-active substances on fish reproductive behavior. For example, Martinovic et al. (2007) conducted a study in which they showed that male fathead minnows exposed to a relatively low concentration of 17β-estradiol, and subsequently placed in a competitive spawning situation with non-exposed males, failed to compete successfully for nesting sites/females. Similar types of results have been reported for other fish species exposed to steroidal estrogens (e.g., EE2; see Part II of this white paper), suggesting that behavioral alterations could be important to consider, especially if they occur at exposure concentrations below those that cause effects on more traditional endpoints such as development and egg production.

Activational Endpoint: Secondary Sex Characteristics

As described above, exposure of developing animals to endocrine-active chemicals can alter phenotypic sex, resulting in skewed sex ratios in populations. These organizational changes observed in secondary sex characteristics (in sexually dimorphic species) and/or gonads typically are not reversible. However, it also is possible to alter secondary sex characteristics, usually in a reversible manner, in sexually-mature fish through exposure to endocrine-active substances. For example, estrogens or anti-androgens can reduce expression of androgen-dependent secondary sex characteristics in males. Similarly, androgenic chemicals can cause female fish to develop male secondary sex characteristics, such as nuptial tubercles in the fathead minnow or elongated anal fins in the Japanese medaka (Seki et al. 2006). Alterations in secondary sex characteristics are much less useful indicators of endocrine-mediated responses in test species, such as zebrafish, with limited sexual dimorphism (Seki et al. 2006).

Alterations in secondary sex characteristics in adult fish can, in some instances, be subtle and somewhat subjective with respect to interpretation. For example, reductions in the status of

existing structures in fish (such as nuptial tubercles in male fathead minnows or anal fin length in male medaka) can be difficult to quantify. However, when there is a *de novo* synthesis of structural characteristics where none previously existed (such as tubercles in female fathead minnows), the response is not only quite specific (in this case to an androgenic MOA), but very easy to detect (i.e., the baseline, control condition is zero).

Although changes in secondary sex characteristics appear to be reasonable mechanistic biomarkers for some endocrine MOA, their utility as a predictor of adverse outcomes (e.g., egg production) is uncertain. Specifically, given our current understanding of fish reproductive physiology/endocrinology, causative links between secondary sex characteristics and gamete quality would be difficult to define. At best, a correlative association may be identified between the two parameters. For example, in studies with the synthetic androgenic steroid 17β-trenbolone, egg production appeared to be reduced at about the same test concentration that caused some degree of nuptial tubercle formation in females (Ankley et al. 2003).

Activational Endpoint: Vitellogenin

Vitellogenin status is probably the most commonly measured endpoint in studies with endocrine-active chemicals in fish. Measurement of the lipoprotein (or its mRNA) is relatively easy via a variety of methods (although most techniques have some degree of species specificity; Wheeler et al. 2005). Production of mRNA (*vtg*) and protein (VTG) in the liver of female oviparous (egg-laying) vertebrates is normally stimulated by activation of the estrogen receptor by endogenous estradiol. The protein is released to the plasma and subsequently deposited in the ovary where it forms a key constituent of developing oocytes. VTG levels in male oviparous animals typically are non-detectable due largely to very low circulating estradiol concentrations; however, males retain the molecular "machinery" in the liver necessary to produce VTG. Hence, exposure to even relatively low amounts of exogenous estrogen or estrogen mimics can stimulate a marked induction of VTG in males. The response not only is specific and sensitive (in part due to a baseline of essentially zero), but relatively sustained after exposure, as the males have no mechanism whereby to clear the protein from their blood.

Although *vtg* and/or VTG induction in male fish is an excellent biomarker of exposure to estrogens (Lattier et al. 2002), the response appears to have little direct (i.e., causative) value in terms of predicting adverse effects on reproduction (e.g., Wheeler et al. 2005). This perhaps should not be surprising given that VTG production in males is not part of any normal physiological pathway. It is possible, however, that correlative relationships between *vtg* and VTG induction in males by exogenous estrogens (such as EE2) and overall effects on fish population status could be derived (e.g., Kidd et al. 2007). This certainly merits additional study, but at present, it appears that the most technically-defensible use of VTG occurrence in male fish is as an indicator of exposure to estrogenic substances in the field and/or confirmation of chemical MOA in laboratory studies.

As opposed to males, VTG has a clear physiological role in females in that the protein is essential to egg production. Concentrations of VTG in females can be reduced by endocrine-active chemicals that directly or indirectly inhibit steroid (ultimately estradiol) production. For example, aromatase inhibitors such as some conazole fungicides decrease steroid production by

inhibiting enzymes involved in steroidogenesis, while other androgenic chemicals like trenbolone decrease steroid production through feedback inhibition in the HPG axis. As a consequence, these classes of endocrine-active chemicals reduce normal VTG production in female fish, thereby reducing fecundity and, ultimately, affecting population status (Miller et al. 2007). Therefore, in the case of females, VTG status may be effectively used as a biomarker both of exposure and effects. Kidd et al. (2007) found that VTG was elevated in female fathead minnows outside of their spawning season. Therefore, elevated VTG in females outside of the spawning season may also be an important measure of stress.

3.3 Pathways and Receptors Beyond the HPG-Axis

As was explained at the outset, this section (Section 3) has a substantial focus on the HPG-axis not because it is the only MOA that is of concern in this document, but because it is currently prominent in both social and scientific arenas. However, it is important to re-emphasize that the use of HPG-active chemicals as a basis for discussion does not imply that this is the only group of CECs of concern with respect to the development of ALC, or the only group for which there may be need for supplementation of the explicit procedures outlined in the *Guidelines*.

As an example, the hypothalamic-pituitary-thyroid (HPT) axis is another endocrine system present in vertebrates that governs important biological pathways and is potentially subject to disruption. Similar to the role of steroid hormones in the HPG axis, actions of the HPT axis are mediated through thyroid hormone, which is involved in the regulation of metabolic activity, energy consumption and muscular activity in adult animals, and the regulation of postembryonic or perinatal growth and development in developing animals, especially in the central nervous system (Chatterjee and Tata 1992). Thyroid hormone is also responsible for the obligatory induction and maintenance of metamorphosis in amphibian and other poikilotherms, and may also play a role in male reproduction (Peterson et al. 1997). Since the actions of thyroid hormone are mediated via binding to highly-conserved nuclear thyroid hormone receptors and modulating transcription of specific genes, disruption of the HPT axis can be disrupted in many ways parallel to those discussed for the HPG axis (Farwell and Braverman 2006), and in doing so, create similar challenges for the development of ALC. Only a few of the developmental actions of thyroid hormones, however, are the result of the direct interaction of the hormone and receptor. Instead, most are indirect via the influence of thyroid hormone on other hormone or growth factors. For example, some of the growth-promoting effects of thyroid hormones on juveniles are indirectly mediated via growth hormone released from the pituitary gland (Chatterjee and Tata 1992).

The amphibian metamorphosis assay is one of the thyroid-relevant *in vivo* screening assays EPA has developed to detect chemicals that interfere with the thyroid hormone system. The assay represents a generalized vertebrate model to the extent that it is based on the conserved structure and functions of the thyroid systems, and thus mirrors some of the assays developed and discussed earlier for the HPG axis. This particular assay is important because amphibian metamorphosis provides a well-studied, thyroid-dependent process which responds to substances active within the HPT axis (Fort et al. 2007). The utility of this and other similar HPT-specific assays for development of ALC is predicated on the principle that the dramatic morphological

changes that occur during post-embryonic development of vertebrates are dependent on the normal function of the HPT axis, and that interference with this process leads to quantifiable effects (Zoeller and Tan 2007).

Other pathways relevant to this discussion could include any of a number of those regulated by different nuclear hormone-type transcription factors, such as the progesterone, glucocorticoid and aryl hydrocarbon (Ah) receptors. Of these the Ah receptor is of particular interest because it has been well studied and is key to the toxicity of several important environmental contaminants such as dioxins and PCBs. Ah receptor agonists are extremely toxic to early life stages of some vertebrates species (e.g., adult fish are at least 10 times less sensitive than early life stages), can induce delayed mortality not captured in short-term (e.g., 96-hour) toxicity tests, and are not very toxic to invertebrates, which lack the receptor (Cook et al. 1993; Mount et al. 2003; Tanguay et al. 2005). Hence, as is true for HPG-active chemicals, knowledge that a contaminant may be an Ah receptor agonist can help focus testing to determine ecological risk (Cook et al. 1993).

Although the previous systems are generally found in vertebrates but not invertebrates, parallel developmental, reproductive, and homeostatic systems exist in invertebrates (Lintelmann et al. 2003) and are most likely just as susceptible to disruption by xenobiotic chemicals. In fact, many pesticides are designed explicitly to disrupt biochemical pathways specific to invertebrates or sub-groups of invertebrates as a means to reduce effects on non-target (vertebrate) organisms. Some endocrine-mediated processes unique to certain taxa of invertebrates include molting, limb generation, diapause, pheromone production, pigmentation and coloration, and metamorphosis. For these processes, the most important endocrine regulators in arthropods are ecdysone and related compounds (ecdysteroids), which are involved in embryonic development, molting, metomorphosis, reproduction, and pigmentation (Lintelmann et al. 2003). Juvenile hormones in insects and methylfarnesoate in crustaceans (both belonging to the class of sexual hormones called terpenoids) are also deemed necessary to mediate the regulatory functions of ecdysteroids (DeFur et al. 1999). Research on the effects of CECs on these systems is still in its early stages, but the parallels with other systems that are susceptible to disruption are clear, and may therefore create similar issues for the development of ALC.

4.0 SUMMARY AND RECOMMENDATIONS

Through its deliberations, the workgroup concluded that the basic framework and conceptual underpinnings of the *Guidelines* apply to CECs as well as other chemicals. Further, the "Good Science" clause of the *Guidelines* provides the flexibility to adopt procedures that will produce a technically rigorous and protective criterion. The focus of this report has been the interpretation and adaptation of the principles set forth in the *Guidelines* with respect to common toxicological characteristics of CECs. In that regard, the workgroup identified a number of possible modifications or alternate interpretations that might aid those developing criteria for CECs to do so in a resource efficient manner that takes best advantage of existing knowledge.

Although some of the recommendations involve increasing flexibility in meeting certain data requirements, the intent is to guide the generation of ALC for CECs that have the same technical rigor as 304(a) criteria developed for other chemicals; these are not methods for "short-cut" criteria. This is a significant point, because an important feature of the *Guidelines* is defining a minimum technical rigor that criteria must have; if insufficient information exists to achieve a minimum level of confidence in the calculated criterion, then criteria should not be derived. The important consequence of this for risk assessors and managers is that when criteria are used to make regulatory decisions, one can have confidence that uncertainty regarding the criterion is not excessive. In other words, criteria derived using the *Guidelines* are often used as both "walk away values" (i.e., there is high confidence that there is little or no risk when exposures are below criteria) and as indicators of risk (implying that effects are likely when criteria are exceeded). If greater uncertainty were allowed in criteria, then the ability to use the values in this way would be compromised.

A negative aspect to establishing a minimum level of information for criteria is that there may be chemicals for which regulatory guidance is needed, but for which toxicological data are insufficient to meet the minimum standards of the *Guidelines*. In such cases, there may still be a need for alternate approaches to derive interim regulatory guidance values on which to base decisions that must be made before sufficient information for a complete water quality criterion can be gathered. While much of the discussion in this report might be useful to inform the development of such an approach, it must be emphasized that developing procedures to derive interim regulatory guidance values based on limited toxicity information is a separate matter and would require considerable additional analysis.

The subsequent sections summarize the issues and recommendations of the workgroup according to the areas of concern identified above.

4.1 Relevance of Acute Toxicity Effect Levels in Setting ALC for CECs

Some CECs may not be acutely toxic, or may only be acutely toxic at environmentally irrelevant concentrations. Thus, if the minimum data requirements for acute toxicity data are not already met by existing data, conducting additional acute tests might be unwarranted. Indication of lack

of acute toxicity in key aquatic species might also warrant direct calculation of the CCC rather than using the FAV/FACR approach, and thus eliminate the need for the full suite of acute toxicity tests normally required.

For a CEC of interest, available information should be reviewed to determine if the CMC would be sufficiently higher than the CCC such that developing the CMC is not needed. Exactly how much data is a risk management judgment, and probably does not have a unique answer. We recommend that the following information be considered when addressing this issue:

- the amount and phylogenetic spread of acute toxicity data available;
- toxicity data from short-term exposures that may not meet the strict definitions in the *Guidelines* of acute toxicity data acceptable for criteria derivation, but from which information on responses to acute exposures can be inferred;
- data on short-term exposures garnered from longer-term exposures;
- information from closely related chemicals thought to have the same MOA that have more robust acute data sets; and
- knowledge of the degree of phylogenetic distribution of the toxicity pathway of concern.

4.2 Defining Minimum Data Requirements in Terms of Taxonomic Coverage

One consequence of dropping acute testing requirements in criteria derivation is the inability to calculate a CCC using the ACR approach, i.e., as the quotient of the FAV and FACR. In addition, for chemicals with large ACRs, it is likely that the mechanisms for acute and chronic toxicity differ (Welshons et al. 2003) and that the sensitivity of different taxa to acute and/or chronic toxicity varies widely. Both of these issues introduce uncertainty into the interpretation of ACRs, and probably make it inadvisable to use the FAV/FACR approach. Under such a circumstance, a prudent approach would generally be to develop a CCC directly from a sufficiently robust set of chronic data, using the procedures outlined in the *Guidelines*. If there is insufficient data from actual toxicity tests to fulfill the MDRs to develop a CCC directly from chronic toxicity data, a reasonable understanding of the toxicological MOA for the chemical may allow inferences as to what taxa (and endpoints) are most likely to be insensitive, such that measured chronic values for those taxa might not be needed. One important consideration in this process is to avoid an excessive number of taxa estimated to be insensitive, relative to those for which actual test results are available, and thus to distort the phylogenetic distribution from that implicit in the MDRs and typical of ALC.

Accordingly, the workgroup recommends that, for chemicals without complete chronic toxicity data sets fulfilling all MDRs, there be an evaluation of whether sufficient information exists to conclude that certain taxa would not be sensitive to the chemical. Given the variation in understanding and availability of data likely to exist for different CECs, it is presumed that at least initial application of this approach would have to be justified on a chemical-by-chemical basis using appropriate scientific judgment. However, lines of evidence that might be applicable to this determination include:

- an in depth understanding of the toxicological (or, in the case of drugs, therapeutic)

MOA;

- information on the basic physiology of other taxa in relation to the MOA;
- toxicity data from chronic exposures or other relevant experiments that do not meet the strict definitions of acceptable chronic data given in the *Guidelines*, but from which information on relative taxon sensitivity can be inferred; and
- information from closely related chemicals thought to have the same MOA that have more robust acute or chronic data sets.

4.3 Use of Non-Resident Species in ALC Development

Historically, EPA has not used data derived from toxicity testing with non-resident species in the actual criteria derivation process. Excluding species simply because they are not resident may be unnecessarily restrictive for the purposes of deriving national criteria, and may actually increase rather than decrease uncertainty. Because ALC are intended to protect "most of the species, most of the time" and use distributions of test data for point estimation, increasing the species representation in the toxicological database should allow better estimation of species sensitivity distributions.

The workgroup recommends that some non-resident species be considered for use in criteria derivation calculations, focusing on those species with widely used and standardized test methods and for which there is no reason to believe would misrepresent the sensitivity of comparable resident species. Furthermore, we specifically suggest accepting data for zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), to reflect international efforts toward data equivalency (Ankley and Johnson 2004). This recommendation pertains to the direct use of chronic toxicity data in the calculation of a CCC as is currently done for resident species. It is worth noting that even non-resident species that are not included in criteria calculations may still provide important information on MOA, sensitivity of endpoints, etc., as expanded on further below.

4.4 Defining Appropriate Chronic Toxicity Data

The *Guidelines* state that acceptable chronic tests for criteria derivation are full life-cycle exposures (egg/birth to egg/birth) for both vertebrates and invertebrates, as well as partial life-cycle (adult to juvenile) and early life-stage (ELS; egg to juvenile) tests for fish. For chemicals for which sexual development/maturation or reproductive effects are of most concern, the allowance in the *Guidelines* for using ELS or partial life-cycle fish tests might need reconsideration. The *Guidelines* already give priority to life-cycle tests when they are available and show greater sensitivity than other tests. However, other information indicating the importance of sexual development and reproduction (from other species, similar chemicals, knowledge of the MOA) might also establish a basis for not considering ELS data and for requiring life-cycle or partial life-cycle tests for fish.

At present, a CCC could be derived for a chemical for which chronic toxicity data for fish are limited to ELS exposures. Because of the importance of sexual maturation and reproduction for

determining the chronic toxicity of chemicals like EDCs, the workgroup recommends strengthening the *Guidelines* such that the chronic toxicity data requirements require that either:

1) Full life-cycle data be available for at least one fish species; or

2) There is a body of experimental information indicating that life processes outside the ELS or partial life-cycle exposure/observation windows would not be important to capturing the important toxicological effects of the chemical.

We note further that although this report is focused on CECs, this recommendation may be important to implement for all chemicals, not just CECs.

Regarding the latter, we recognize that there may be circumstances where the information that shows the sensitivity of different life stages comes from studies that, while scientifically valid, for some reason do not meet all the requirements of a valid life-cycle test as defined in the *Guidelines*. Alternatively, there may be data from experiments that violate other requirements of acceptable toxicity tests under the *Guidelines*, but still provide insight into sensitive exposure periods or life processes. Even though chronic values from such data may not be used directly to calculate a CCC, it seems a reasonable use of such data to evaluate the question of where in the life-cycle there are important windows of exposure and/or effect, and how that impinges on criteria derivation.

It may also be that meaningful chronic toxicity data could be developed from exposures that have a structure different from the life-cycle, partial life-cycle, and ELS protocols defined explicitly in the *Guidelines*; e.g., a short-term (21-day) reproduction assay with the fathead minnow (U.S. EPA 2001) or a multi-generational study – see example for EE2 reported in Nash et al. (2004). While defining such alternate protocols is beyond the scope of this document (see Ankley and Johnson 2004 for more detail), we recognize the potential for such a situation and leave it to appropriate implementation of the "Good Science" clause to allow for inclusion of such alternative test protocols as surrogates for chronic toxicity data, most likely in addition to, rather than instead of, data from life-cycle toxicity tests.

4.5 Selection of Effect(s) Endpoints Upon Which to Base ALC

Although chronic criteria typically are based on direct effects on reproduction, growth, and survival, there may be other endpoints indirectly related to these responses that could be useful for criteria derivation. The selection of endpoints appropriate to the derivation of ALC must be tied to the narrative intent of the overall *Guidelines*. The stated goal of ALC is to "protect aquatic organisms and their uses." While the exact meaning of "protection" is not defined, there is considerable discussion in the *Guidelines* document that makes clear that protection does not mean the prevention of any measurable biological effect in any organism. Instead, there is discussion of endpoints that are "biologically important" and prevention of "unacceptable effects"; this implies that in the context of criteria there are effects that are "biologically unimportant" and/or levels of effect that are "acceptable."

Chronic test data and other data should be examined to determine whether, for the specific chemical or MOA, endpoints beyond those traditionally used for criteria derivation may have intrinsic "biological importance" and therefore could be used as a basis for defining threshold of effect (e.g., sex ratio). Specifically, in the context of EDCs:

- Other "endocrine-sensitive endpoints" (e.g., VTG, testis-ova) should be examined to determine whether they can be relied upon as definitive indicators of other biologically important endpoints (e.g., reproduction), with the idea that they may be incorporated into calculation of the criterion. Important sources of this information would include full life-cycle tests in which these other endpoints were measured alongside traditional chronic endpoints, and may include tests with other chemicals with the same MOA (e.g., E2 for EE2).
- If endpoints, such as VTG or testis-ova, are used as direct or indirect indicators of effect, it is critically important that the baseline condition (e.g., variation during normal development) be understood sufficiently to define when changes are biologically meaningful.
- Selection of appropriate endpoints (and their associated effect thresholds) may, in some instances, transcend "biological importance" (the focus of the *Guidelines*) to reflect societal concerns (e.g., physical appearance of wild-caught fish).

4.6 Involvement of an Expert Panel

As becomes clear from the preceding issues, development of appropriate criteria for CECs may be unusually dependent on technical interpretations of a wide range of toxicological information pertinent to specific chemicals. One of the recommendations from a SETAC Pellston workshop (Mount et al. 2003), consistent with much of the above, was that expert panels be used to provide professional judgment during stages of the problem formulation and data interpretation associated with criteria development, particularly for chemicals with specific MOA. The involvement of the panel would "ensure consideration of other existing data for the chemical of concern, enable a significant degree of up-front technical input, and provide a level of peer review that should facilitate wider and more ready acceptance of the recommended criteria." The workgroup agrees with this recommendation and suggests that it be incorporated into criteria development of CECs.

To maximize effectiveness, this panel should be convened very early in the criteria development process such that it will be able to assist in problem formulation, identification of important data, and scoping of particular issues that will be important. We envision these panels as being formed around specific chemicals, or groups of chemicals with a similar MOA, in order to access the most specialized expertise available.

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WHITE PAPER

AQUATIC LIFE CRITERIA FOR CONTAMINANTS OF EMERGING CONCERN

PART II

ILLUSTRATION OF RECOMMENDATIONS USING DATA FOR 17α-ETHYNYLESTRADIOL (EE2)

Prepared by the
OW/ORD Emerging Contaminants Workgroup

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NOTICE

THIS DOCUMENT IS AN INTERNAL PLANNING DOCUMENT

It has been prepared for the purpose of Research & Development Planning.
It has not been formally released by the U.S. Environmental Protection Agency and should not at this stage be construed to represent Agency guidance or policy.

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1.0 INTRODUCTION

In Part I of this white paper, toxicological characteristics of some contaminants of emerging concern (CECs) important to the derivation of ambient water quality criteria for aquatic life (aquatic life criteria, ALC) were described, and recommendations were made to facilitate ALC derivation for these chemicals. In Part II of this white paper, toxicity data for a model CEC, 17α-ethynylestradiol (EE2), are used to further illustrate and explore those recommendations. Ethynylestradiol was chosen as a model compound for a several reasons. First, it possesses many of the toxicological characteristics described in Part I, and sufficient toxicity data exist to allow evaluation of the principles underlying the Part I recommendations. Second, toxicological effects of EE2 have been found both in the laboratory, the source of toxicological data for criteria development, and in the field, where criteria are used to enforce the regulatory authorities of the Clean Water Act. Finally, there is interest in deriving an EE2 ALC, and using EE2 as a basis for discussion should help advance that goal. While acknowledging that interest, it is important to note that the data and discussion presented are not intended to represent the formulation of an actual ALC, and potential ALC concentrations should not be inferred. The information from the ecotoxicological literature used here is for illustrative purposes and should not be considered as comprehensive, nor have all the data been fully examined for quality and applicability to ALC development.

The synthetic estrogenic steroid EE2 is the active pharmacological component of most oral contraceptives, and acts as a potent estrogen receptor agonist in vertebrates. After use and excretion of the contraceptive, domestic sewage treatment plant (STP) effluents become the primary source of EE2 entering the aquatic environment (Damstra 2002). Kolpin et al. (2002) found EE2 in 5.7% of 139 streams monitored in the U.S. While the concentrations of EE2 in Kolpin et al. (2002) have been debated (Ericson et al. 2002; Till 2003), other studies have noted concentrations ranging from 0.1 – 5.1 ng/L in surface waters (as reviewed by Campbell et al. 2006). Overall, it is somewhat uncertain at this time how high environmental concentrations of EE2 may be. Reliable analytical methods for the detection of EE2 have not been in existence very long, nor have they been widely validated in independent multi-laboratory studies. Some modeling efforts by the pharmaceutical industry indicate that based on the level of production and use of EE2 in the U.S., concentrations found in effluents should be less than 1 ng/L (Anderson, P.D. and D'Aco, V, personal communication, 2008). Complicating assessment of the possible risk of EE2 is the fact it co-occurs in STP effluents with the natural steroid hormones estradiol and estrone, though EE2 is generally found at lower concentrations. These three estrogens reportedly account for the majority of the estrogenic activity present in domestic wastewater effluents (Desbrow et al. 1998; Snyder et al. 2001), but EE2 is the most potent and resistant to degradation of the three (Nash et al. 2004; Gross-Sorokin et al. 2006). Data collected from fish and surface waters downstream of STPs over the past decade have implicated steroidal estrogens as the primary constituents in domestic effluents leading to the occurrence of intersex fish (Gross-Sorokin et al. 2006).

The remainder of this part of the white paper consists of a brief description of some relevant acute and chronic toxicity data available for EE2 (Section 2) and the evaluation of these data with respect to the recommendations made in Part I (Section 3).

2.0 DESCRIPTION OF THE ACUTE AND CHRONIC TOXICITY DATA SUMMARIZED FOR EE2

Acute and chronic toxicity data were identified via a literature search and review of relevant articles from EPA's ECOTOX database in April 2007. This list of potentially useful articles was supplemented with a few additional reports and articles as they became published or available. Only those studies with EE2 effect data on individual aquatic organisms or their populations were retained. For this particular effort, all endpoints expressing effects of EE2 at the whole animal and cellular levels were initially considered. Because the EE2 dataset is relatively large, and because many studies report more than one endpoint of possible consideration for ALC development, the data have been broadly summarized in an appendix (Appendix A). The tables comprising Appendix A are organized by endpoint, and include separate tables for endpoints typically used to derive ALC (survival, growth and reproduction) as well as for other endpoints relevant to the estrogenic mode of action of EE2. Table A.1 contains the data available on the acute (lethal) toxicity of EE2 to aquatic animals. This table is followed by others containing the chronic (long-term) effects of EE2 on survival (Table A.2) and growth of aquatic animals (Table A.3). Tables A.4 through A.9 present data directly (fecundity, fertility) or indirectly (sex reversal, intersex, sexual behavior, vitellogenin) related to the effects of EE2 on reproduction. Finally, Table A.10 presents a summary of the significant effects of EE2 on aquatic animals based on other potentially relevant endpoints. *In vitro* effects were not considered in the data analysis.

Within each table in Appendix A, data are first separated by studies where significant effects were observed, and then by studies where significant effects were not observed (i.e., where no effect was observed at the highest concentration tested). Each table in the appendix combines data for aquatic vertebrates and invertebrates for both freshwater and saltwater species, the latter designated by asterisks. All tables are organized by increasing effect concentrations, and all chronic effect endpoints are as reported by the authors.

Many studies of the effects of EE2 on aquatic organisms did not use standard toxicity test protocols, particularly those measuring sublethal responses. This is probably due in part to these studies having been designed for purposes other than ALC development, such as exploration of toxicity mechanisms, identification of sensitivity windows, bioassay development, etc. Adequate quantification of effect concentrations is also difficult for some of these studies because of the use of widely spaced treatment concentrations and by problems with analytical detection of exposure concentrations near the threshold for reproductive effects. While the results from such studies might limit their use in ALC development according to the definitions in the *Guidelines*, they were included in this document because they may inform other aspects of criteria derivation, as explained in general terms in Part I and in detail in the sections that follow.

3.0 EE2 DATA EVALUATION AND CONSIDERATIONS FOR ALC DEVELOPMENT

This section considers the application of the data in Appendix A toward criteria derivation in the context of the several areas of concern and general recommendations identified in Part I of this white paper.

3.1 RELEVANCE OF ACUTE TOXICITY EFFECT LEVELS IN SETTING ALC FOR EDCS

One of the recommendations from Part I of this document was to determine whether the acute sensitivities of aquatic organisms to a chemical of interest are sufficient, relative to chronic sensitivity and expected exposures, to warrant derivation of a criterion maximum concentration (CMC) under the *Guidelines* procedures. This is especially important if there is not sufficient acute toxicity data to meet the minimum data requirements of the *Guidelines*, in order to avoid wasting resources on unnecessary additional testing. EE2 provides a good example of a chemical having insufficient acute toxicity data to derive a CMC according to *Guidelines* procedures, but enough data to demonstrate that deriving a CMC is not necessary.

Table 3.1 provides information on GMAVs that might be considered in CMC derivation. These values were derived from Table A.1 for any tests meeting *Guidelines* requirements, including "greater than" values indicative of the highest tested concentration eliciting less than 50% mortality. For genera without such acceptable tests, EC50/LC50s from Table A.1 for tests of 24 h duration and from Table A.2 for tests up to 30 days were also used. The EC50/LC50 values for these longer tests are designated as "greater than" values to indicate the expectation that acute EC50/LC50s would be higher. Values for medaka and zebrafish are included in accordance with the recommendation from Part I of this document that some latitude be adopted regarding species not resident to North America. Acute tests with embryos, not usually included in CMC calculations, are also included here because they suggest greater sensitivity of this life stage.

Table 3.1. Potential GMAVs for Application to EE2 CMC.

Genus	GMAV (ng/L)	Comments
Freshwater		
Rana	>760,000	14-d test
Gammarus	>840,000	10-d test
Medaka	>1,000,000	
Danio	1,700,000	
Ceriodaphnia	1,800,000	
Hydra	3,800,000	
Sida	>4,100,000	24-h test

Genus	GMAV (ng/L)	Comments
Daphnia	>5,000,000	
Chironomus	9,100,000	24-h test
Saltwater		
Lytechinus	30,000	Embryo
Strongylocentrotus	30,000	Embryo
Acartia	88,000	Embryo
Tisbe	>100,000	21-d test
Acartia	1,100,000	
Neomysis	1,200,000	

The data summarized in Table 3.1 show several deficiencies in meeting the minimum data requirements for deriving a CMC under the Guidelines. For freshwater application, only four genera, rather than the minimum of eight, meet the acute test requirements, even if the prohibition for non-resident species is ignored. If the shorter and longer tests are included, the requirement of at least eight genera is met, but the requirement for a salmonid fish is not. Even if this requirement is also ignored, two of the lowest four genera are "greater than" values, whereas CMC calculations can only be made if the four most sensitive genera have definite values ("greater than" values are permitted only for more tolerant genera.) For saltwater, there are even greater deficiencies in meeting the minimum data requirements.

Although these data are insufficient for deriving CMCs, they do provide ample evidence that a CMC is not needed and that it is unnecessary to conduct further tests to meet the minimum data requirements. For freshwater, there is still a rather broad taxonomic representation, including three vertebrates from two different classes, four crustaceans from two orders, and a third phylum. The acute LC50s/EC50s are consistently near and above 1 mg/L, several orders of magnitude above both the most sensitive chronic endpoints (Tables A.4 – A.9) and the highest environmental concentrations that organisms might be exposed to. The saltwater data do show greater sensitivity for the embryonic stages of some genera, but whether this reflects a lifestage or taxa sensitivity issue, these LC50s/EC50s are still four orders of magnitude above the most sensitive chronic endpoints and environmentally-relevant exposures.

3.2 USE OF NON-RESIDENT SPECIES IN ALC DEVELOPMENT

Under the *Guidelines*, toxicity values from aquatic species not resident to the contiguous 48 United States, Alaska, or Canada are excluded from ALC derivation. One of the recommendations in Part I of this white paper is that this prohibition be relaxed and that data for non-resident species be allowed where deemed suitable, especially for species such as medaka and zebrafish which have become standard test organisms commonly used worldwide. Any tested species, whether resident or not, serves as a surrogate for estimating a sensitivity

distribution relevant to assessing risks in a variety of aquatic communities with a multitude of untested species. Therefore, the issue here is whether a non-resident species can serve as a reasonable surrogate for assessing the sensitivity of untested resident species. The use of such species would still be contraindicated if there is reason to believe they are significantly more or less sensitive than resident species.

The data in Appendix A support the use of medaka and zebrafish data in criteria calculations. Although there are no resident fish species with which to compare the acute sensitivities of medaka and zebrafish (see Table 3.1 and Table A.1), their lack of acute sensitivity is consistent with that of resident amphibians and invertebrates in the available data. The sensitivities of these fish species for long-term survival (Table A.2), growth (Table A.3), and reproduction (Table A.4) are interspersed with those of resident fish species, so there is no indication of either substantially higher tolerance or sensitivity to contraindicate their use. This is also generally true of the other endpoints summarized in Appendix A. The similarity among fish species of different geographic origins is not surprising, since the MOA of EE2 involves receptors and pathways that are highly conserved among vertebrates. If similar trends are seen in the data once they are thoroughly examined for quality and applicability to ALC development, data from these non-resident species should be included in criteria development.

The data in Appendix A also underscore pragmatic advantages of including non-resident species in criteria development. Medaka and zebrafish provided a large fraction of the available data regarding EE2 effects on fish. Removing them from the dataset simply because they are not resident would limit information on the distribution of species sensitivity and may actually increase rather than decrease uncertainty regarding resident species. Another use of data from non-resident species could be to assist in extrapolations of information across species, chemicals, and endpoints. For example, life-cycle tests with medaka could be used to evaluate whether early life-stage or partial life-cycle tests with resident species should or should not be accepted in criteria calculations for specific classes of chemicals with a defined MOA. The relationship of reproductive effects in non-resident fish (Table A.4) to other endpoints (Tables A.5-A.9) could also be used to determine how to apply information on these other endpoints for resident species lacking direct toxicological information on reproduction.

3.3 MINIMUM DATA REQUIREMENTS REGARDING TAXONOMIC COVERAGE

As discussed in section 3.1, deriving a CMC for EE2 is not useful because acutely-toxic concentrations are so much higher than both chronic effects concentrations and expected environmental concentrations. In addition, developing a CMC would require additional acute toxicity tests to meet the minimum data requirements (MDRs) specified in the *Guidelines*. Without a CMC, the criterion continuous concentration (CCC) must be calculated directly from the available data, rather than through extrapolation using an acute to chronic ratio (ACR); this is probably not advisable anyway for such large ACRs. Since the ACR method is moot, the *Guidelines* calculation procedures for the CCC require that there be sufficient chronic toxicity tests to satisfy the MDRs for estimating the fifth percentile of the chronic database. For

freshwater criteria, these MDRs include a species from: the family Salmonidae; a species from a second family in the class Osteichthyes; a species from a third family in the phylum Chordata; a planktonic species from the Class Crustacea; a benthic species from the Class Crustacean; a species from the Class Insecta; a species from a phylum other than Chordata or Arthropoda; and a species from an order of insects or a phylum not otherwise represented.

Few existing ALC have chronic data that meet the MDRs, and this will likely be true of CECs as well. Significant expense would be incurred conducting new chronic tests to satisfy all the requirements. As recommended in Part I of this white paper, because only the four most sensitive genus mean chronic values (GMCVs) are used in the criteria calculations, chronic testing requirements for a taxon needed to meet an MDR should be waived if there is sufficient information to conclude that this taxon is more tolerant than the four most sensitive genera. A value (or values) for this taxon would still be included in the data set, but its GMCV would simply be specified to be greater than the fourth lowest GMCV.

Table 3.2 lists chronic values for the toxicity of EE2 to various freshwater genera to illustrate data that might be included in freshwater criteria calculations. These chronic values were obtained from Tables A.2-A.4, using *Guidelines* data selection procedures where possible, but also included some additional data to support discussion of how certain data deficiencies might be addressed. For invertebrates, the *Guidelines* require life-cycle tests that include reproductive endpoints, but if that type of test was not available, then other tests are reported here, with their limitations noted. For fish, the *Guidelines* preference order of life-cycle, partial life-cycle, and early life stage (ELS) was followed, but other tests were also reported as needed for illustrative purposes, with their limitations also noted. For all genera, the most sensitive endpoint among chronic survival, growth, and reproduction was selected, which was from the reproduction data of Table A.4, except for *Chironomus* (for which development from egg to pre-emergence was tested and the effects concentration was from Table A.3). Each chronic value (CV) was calculated as the geometric mean of the reported no observed and lowest observed effect concentrations (NOEC and LOEC) for an adverse effect. When the LOEC was the lowest exposure concentration, a "less-than" concentration is reported for the CV and, when the NOEC was the higher exposure concentration for insensitive species, a "greater-than" concentration is reported. As explained in Sections 1 and 2, these data are still under review and subject to modification. The specific values here should not be misconstrued as final, but rather as examples to illustrate trends and indicate needs that support the recommendations being addressed here.

Table 3.2. Potential Chronic Values for Application to EE2 CCC.

Genus	CV(s) (ng/L)	Notes
Danio	0.6, 1.5, <1.1	Life-cycle tests; for 1.5 ng/L CV, there was a 9-fold difference between LOEC and NOEC and the LOEC was a 100% effect
Pimephales	<0.32, 1.5	Life-cycle tests; for <0.32 ng/L CV, LOEC showed reduced fertilization but increased egg production so total reproduction not adversely affected; for 1.5 CV, 4-fold difference between NOEC and LOEC
Oryzias	3.2	F_0 from 1 d through spawning; 10-fold difference between NOEC and LOEC
Oncorhynchus	<16	Adult exposure only; fertilization success only endpoint examined
Potamopyrgus	50	Adult exposure only; embryo production over 9 wk test
Gammarus	>7,600	Population size over 100 d test; increased population size at 760 and 7,600 ng/L
Daphnia	45,000	5-fold difference between NOEC and LOEC
Tisbe	>100,000	Saltwater copepod included to further indicate arthropod insensitivity
Chironomus	320,000	Larval growth and molting schedule only; did not include emergence and reproduction
Brachionus	800,000	Intrinsic rate of population increase over 72 hr test

The data in Table 3.2 indicate high sensitivity of vertebrates to EE2. Significant reproductive effects in the life-cycle tests for zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) occur at concentrations near and perhaps below 1 ng/L. Although the chronic test for medaka (*Oryzias latipes*) did not cover the entire life cycle, it included life stages likely important for reproduction and indicated a sensitivity similar to zebrafish and fathead minnow. For rainbow trout (*Oncorhynchus mykiss*), a more limited exposure addressing only effects on fertilization suggests that reproductive effects on this species should also be present in the low ng/L range. However, the absence of a definite toxicity value for rainbow trout will be an important impediment to criteria calculations, both for leaving an MDR unsatisfied and for being one of the four most sensitive genera in this set. Actual criteria development will require a decision whether to (a) require more information for this species, (b) use other information to help estimate rainbow trout sensitivity or (c) justify setting the MDR aside (see Section 3.5).

The invertebrate data in Table 3.2 indicate lower sensitivity, especially for arthropods (*Gammarus, Daphnia, Tisbe, Chironomus*) and rotifers (*Brachionus*). Some data, like the *Chironomus* test, fail to satisfy the *Guidelines* requirement for a life-cycle test and the copepod *Tisbe* is a saltwater species included here only to reinforce conclusions about arthropod sensitivity. Also, the tests for *Gammarus* and *Brachionus* are not standard life-cycle tests, but could be considered to satisfy *Guidelines* requirements because exposures span a life cycle and

include reproductive effects. The snail (*Potamopyrgus*) toxicity test showed moderate sensitivity, although still about an order of magnitude less than the fish, and also does not involve a full life-cycle test.

These data demonstrate the potential for a situation in which the GMCVs for taxa reasonably expected to be insensitive do not need to be quantified. For example, although the *Chironomus* test was not a full life-cycle test and thus could not fully define the GMCV under *Guidelines* requirements, it indicates such a degree of insensitivity for growth and development, such that it can be reasonably presumed that a full life-cycle test would still show much less sensitivity than the vertebrates, especially because other arthropods are observed to be similarly insensitive. Likewise, the snail test, although not for a full life-cycle, involved the effects of long exposures on reproduction, and can be argued to be sufficiently less sensitive than fish reproduction so that it would not reasonably be expected to be among the four most sensitive genera if a life cycle test was conducted. These inclusions, along with the data for *Daphnia*, *Gammarus*, and *Brachionus*, satisfy the *Guidelines* MDRs for invertebrates, and would allow an ALC to be calculated from the four sensitive vertebrate genera, provided the value for the rainbow trout was resolved.

Assessing that taxa are likely to be insensitive could involve other lines of evidence, especially for CECs with more limited chronic toxicity data than EE2. Tests involving endpoints such as those in Tables A.5-A.10 could be used to establish that certain taxa are sufficiently less sensitive than others to preclude the need for tests on their chronic survival, growth, and reproduction (Tables A.2-A.4), the endpoints typically used in ALC development. Information from other chemicals might also be used, such as using the insensitivity of arthropods to EE2 to preclude testing this taxonomic class with chemicals with the same MOA. Such a strategy could be used to help the evaluation of EE2, particularly regarding the snail sensitivity. For example, the sensitivity of this or similar species relative to that of vertebrates for other chemicals could be used to strenghten a conclusion that they are less sensitive to EE2 than are fish.

3.4 DEFINING APPROPRIATE CHRONIC TOXICITY DATA

As discussed in Part I of this white paper, characteristics of some CECs require that careful consideration be given to the selection of chronic toxicity data appropriate for ALC development. Specifically, the use of data from early-life stage (ELS) or partial-life cycle (PLC) exposures as estimates of life-cycle chronic effect thresholds is inadvisable for chemicals whose MOA would result in biological effects for which critical periods of induction and/or expression would lie outside the exposure/observation window provided by the test procedure.

An examination of data specifically for EE2 provides evidence to support emphasis on full life-cycle exposures for determining the chronic toxicity of EE2. Länge et al. (2001) conducted a full life-cycle chronic exposure with fathead minnows which were exposed from fertilized eggs (F_0) through maturation, spawning, and early-life stage development of the F_1 generation. Nominal exposure concentrations of EE2 were 0.2, 1, 4, 16, and 64 ng/L (for convenience, nominal concentrations are used in this discussion as the important point is relative endpoint sensitivity

rather than absolute concentrations inducing effects). As part of this exposure, measurements of growth (as length) and survival were made at 28 days post hatch (dph) which would correspond to the end of a standard ELS exposure with fathead minnows. At 28 dph, there were no effects on survival. The length endpoint showed a 16% reduction at 64 ng/L, a smaller but significant reduction of 6% at 16 ng/L, and no effect at 4 ng/L. Accordingly, the NOEC and LOEC for an ELS test with EE2 would have been 4 and 16 ng/L, respectively, and an EC20 based on length would be >64 ng/L. However, as exposure continued throughout the life cycle, pronounced effects were observed for other endpoints at lower exposures. There was no reproduction at all in fish exposed to 4 ng/L, and a trend, though not significant, toward lower reproduction at 0.2 and 1 ng/L. Other significant effects observed included a 16% reduction in weight of adult female fish at 1 ng/L after 301 d exposure, and 5 to 10 percent reductions in weight of F_1 offspring at 28 dph, though the authors questioned the biological significance of the F_1 growth effects. Regardless, the clear indication is that life-cycle exposure showed substantially greater sensitivity to EE2 than was evidenced from ELS endpoints alone. This was much larger than the factor of 2 difference generally found for other chemicals by McKim et al. (1978).

A similar conclusion can be drawn from the study of Parrott and Blunt (2005). This involved exposure from fertilized egg through reproduction, including measures of fertilization success (but not ELS development) in the F_1 generation. Exposure was to nominal concentrations of 0.32, 0.96, 3.2, 9.6, and 32 ng/L EE2. Measurements of survival and growth at 30 dph showed no effects (NOEC $\geq$ 32 ng/L). However, continuation of exposure through adulthood showed no reproduction in the 3.2, 9.6 and 32 ng EE2/L treatments, and all fish in these treatments were phenotypic females. There was also suggestion of effects on fertilization success at 0.32 and 0.96 ng/L, although interpretation of these effects is complicated by an increase in number of eggs produced in these same treatments, such that the total number of fertilized eggs was not as dramatically affected. Regardless, the message relative to definition of chronic sensitivity is the same in that effects were apparent after life-cycle exposure at concentrations well below those that would be expected to show effects in an ELS test.

Additional comparisons can be extracted from the work of Wenzel et al. (2001), who conducted a multi-generational study of zebrafish exposed to EE2 concentrations from 0.05 to 10 ng EE2/L. Observations of survival and length of exposed fish showed no effects at 21 and 42 dph (NOEC $\geq$10 ng/L). However, with continued exposure, a variety of effects were observed around an EE2 test concentration of 1 ng/L, including effects on adult length, time to spawning, egg production and fertilization. As for the fathead minnow studies, survival and growth measured during the period comparable to an ELS study were far less sensitive to EE2 exposure than were endpoints measured in full life-cycle studies (Tables A.2, A.3, A.4).

The reason for these differences between ELS and full life-cycle tests is obvious when one considers the MOA for EE2, which interferes with sexual differentiation, development, maturation, and spawning. Because the endpoints measured in ELS tests are limited to survival and growth, and because the effects of EE2 on sexual differentiation are not apparent (at least not at a gross morphological level) in the tested species by 28-30 dph, the ELS test is comparatively insensitive to toxicity mediated through an estrogen receptor signaling pathway. It is interesting

to note that even though a standard ELS test is relatively insensitive to detecting the effects of EE2 exposure, other work has shown that key windows of exposure do in fact occur during the ELS exposure window. Van Aerle et al (2002) demonstrated that larval fathead minnows exposed to EE2 only during brief windows during early development (e.g., 10-15 dph) showed altered sexual development of male fish at 100 dph, including the development of an ovarian-like cavity and changes in the distribution of testicular cell types (Table A.6). The issue for interpreting chronic toxicity data is that, even though effects may be induced during ELS exposure, they are not expressed unless exposed fish are observed later in sexual development.

This latter observation also has implications for the suitability of PLC tests for detecting the effects of EE2 or other chemicals acting through a similar pathway. As discussed in the *Guidelines*, PLC tests are acceptable chronic tests for fish species that require more than one year to reach sexual maturity, such as the common species of trout. PLC tests are to begin exposure "with immature juveniles at least 2 months prior to active gonad development, continue through maturation and reproduction, and end not less than 24 days (90 days for salmonids) after the hatching of the next generation." If salmonids (or other species for which PLC tests might be conducted) were to express effects from larval exposure to EE2 as observed by Van Aerle et al. (2002) for fathead minnows, one would expect that a PLC exposure would not be as sensitive as a full life-cycle exposure. That is, even though a PLC test includes the observation periods shown to be sensitive in full life-cycle exposures, it might not include the exposure windows important to inducing chronic effects on sexual differentiation and development.

While the rationale for emphasizing full life-cycle chronic tests has clear grounding in the MOA for EE2, it has practical implications in terms of the fish species likely to be tested. Most species for which life-cycle chronic tests are most commonly conducted (primarily fathead minnow, zebrafish, medaka, flagfish, and sheepshead minnow), are small fish that develop rapidly and are continuous spawners (as opposed to annual spawners like rainbow trout or bluegill sunfish). Whether or not these life history traits influence sensitivity to EE2 is unknown, but because of the investment necessary to conduct true life-cycle exposures with annually spawning fish that take much longer to develop, it may be unlikely that comparative data will be developed. Better understanding these implications is a worthy subject for future research.

Finally, there are some scattered indications in the literature for trans-generational effects of EE2 exposure. As mentioned above, Länge et al. (2001) found small effects on growth in the F_1 generation that were not observed after comparable exposure of the F_0. Wenzel et al. (2002) also report some suggestions of growth inhibition in subsequent generations at exposure below those causing such effects in the first exposed generation (Table A.3). The mechanisms by which such effects might occur are not clear, nor are their implications (in fact, Länge et al. actively dismiss them as being biologically unimportant). At this point, it does not seem that the evidence for trans-generational effects is sufficient for requiring their inclusion in the definition of an acceptable chronic test, but the potential for the existence and importance of trans-generational effects should be re-evaluated in the future as additional data become available.

3.5 SELECTION OF EFFECT(S) ENDPOINTS UPON WHICH TO BASE ALC

Aquatic studies with EE2, particularly those using fish, have measured a variety of endpoints not traditionally used for criteria derivation, including reproductive behavior, abnormal sex ratios, changes in secondary sexual characteristics, altered histopathology (typically gonadal), changes in steroid hormones, and modifications in the expression (or activity) of a variety of proteins/enzymes. Many of these endpoints were evaluated because they are known (or hypothesized) to be responsive to estrogenic MOA, and not because the intended result was the quantitative assessment of risk. Among the challenges in using data from these types of mechanism-based endpoints is that such measurements are seldom standardized or straightforward in their interpretation. For example, alterations in behavior are difficult to objectively quantify, it is challenging to accurately measure steroid hormone concentrations in small fish, and the capability of measuring gene expression or enzyme activity can be quite lab/method-specific. A second source of uncertainty in using most of the mechanism-specific endpoints evaluated in EE2 studies is a lack of knowledge concerning the functional relationship between changes in endpoints and responses of primary concern for risk assessment, such as survival, growth and reproduction. Even in considering these challenges related to measurement and interpretation, however, there are a handful of mechanistic endpoints/responses that exhibit utility for supporting ALC derivation for EE2 (or other xenobiotic compounds with estrogenic activity). Three of these are discussed in greater detail below.

A frequently measured mechanism-specific endpoint in fish exposed to EE2, is induction of vitellogenin mRNA (*vtg*) or expression of circulating vitellogenin protein (VTG) in males (Table A.9). The most attractive attribute of this endpoint is its specificity for an estrogenic MOA, since there are no other chemically linked biological phenomena known to consistently activate the vitellogenin gene or elevate vitellogenin protein in male fish. Further, since the vitellogenin gene is quiescent in male fish, which implies a zero baseline of vitellogenin, the response is unambiguous with regard to exposure. Additionally, this exposure mediated induction of *vtg* is sensitive to low levels of exogenous estrogen. Because vitellogenin protein has been frequently evaluated in fish studies, accurate methods of measurement (including several commercial kits) are available for many fish species, including the small fish models for which much of the EE2 chronic toxicity data exist. Given these attributes, male VTG has and should continue to be a very useful endpoint for monitoring the occurrence of estrogenic chemicals (including EE2) in the environment. A major drawback to using male-specific circulating protein to assess exposure and risk of EE2 (or other estrogens), including the derivation of ALC, is the lack of an established functional linkage between expression of the protein and adverse endpoints related to early development or reproduction (Wheeler et al. 2005). This is in large part due to the fact that VTG plays no physiological role in male reproductive processes. As such, any associations that might exist between VTG induction in males and reproductive success is likely more correlative than causal.

Despite the fact that the appearance of VTG in males appears not to be a robust predictor of adverse effects on reproduction, the response could nonetheless play an important role in reducing the uncertainty of ALC for EE2, or the development of ALC for other chemicals which

might be estrogenic. From Table 3.2 above, it is apparent that data from life-cycle tests with fish would be appropriate (and critical) to setting the final ALC for EE2 and, by extension, other estrogens. Hence, knowledge that a less well-studied chemical than EE2 induces VTG in males could be used to help identify those instances when one (or more) life-cycle fish assay(s) would be recommended for generating robust data for ALC derivation. Another possible use of protein data, that could have more direct applicability to developing an ALC for EE2, involves use of the endpoint as a basis for evaluating relative species sensitivity. Specifically, reproductive data suitable for an EE2 ALC are largely from three species: fathead minnow, medaka and zebrafish (Table 3.2); however, there are studies with numerous species that have evaluated the ability of EE2 to induce vitellogenin mRNA and protein in males. Provided that a common dose metric could be established across these studies, a dataset could be developed to provide an indication of the relative sensitivity distribution of fish species to EE2, in addition to other estrogens. This would enable a direct comparison of values along the continuum of estrogen sensitivity for those fish species wherein chronic data exist (based on VTG induction) and, as such, could provide a quantitative indication of uncertainty for a proposed EE2 criterion.

There are two mechanism-specific endpoints that have been measured in a number of EE2 studies that might, with additional research and analysis, have a direct bearing on criteria derivation: alterations in sex ratio (i.e., generation of genotypic males with a female phenotype) and the occurrence of intersex/testis-ova (Tables A.5, A.6). As opposed to VTG induction in males, the functional linkage between skewed sex ratios or abnormal gonad development and reproductive success in fish, at both the individual and population levels, is readily apparent. Specific endpoints, however, can be difficult to measure. For example, detailed histological analyses are needed to identify and, especially, quantify testis-ova. To detect an alteration in sex ratio, a genotypic marker of gender (available in medaka but not fathead minnow or zebrafish) or a relatively large representative sample is required to reliably detect chemically-induced changes within a proportion of males and females in a population. Probably more difficult than measurement of the endpoints is definition of the quantitative linkage between changes in sex ratio or occurrence of testis-ova and effects on reproductive success for individuals and populations. For example, unless one assumes that any deviation in sex ratio from the norm (e.g., 1:1) is adverse, it is necessary to know (in the case of estrogenic effects) the magnitude of shift in respective gender numbers that is likely to result in cases where fewer young are produced. Similarly, it is probable that some degree of testis-ova would not be considered adverse in terms of reducing reproductive success, especially considering that the condition can exist at some degree, even in control animals (Grim et al. 2007). It is certain that at some level of manifestation, the condition will impair gonad function sufficiently such that acceptable levels of normal sperm cannot be produced. The frequency of this phenomenon, however, is currently unknown for any fish species. Definition of this relationship would support use of testis-ova occurrence in fish not only for prospective assessments (like criterion derivation), but in environmental monitoring studies focused on chemicals with an estrogenic MOA.

At present, uncertainties regarding measurement and interpretation hamper use of data from any of the mechanism-specific endpoints mentioned above as a basis for derivation of an ALC for EE2. Eith appropriate research, however, induction of vitellogenin in males, changes in sex

ratios and occurrence of testis-ova, all have the potential to contribute insights to different facets of quantitative risk assessment for estrogenic chemicals, including derivation of ALC. There is one noteworthy additional observation relative to use of non-traditional endpoints for an EE2 ALC. Several fish life-cycle studies using EE2 have been conducted in which typical measures of reproductive success (e.g., fecundity, fertility) have been made in conjunction with induction of VTG, sex ratio and/or testis-ova data (e.g., Länge et al 2001; Wenzel et al. 2001; Nash et al. 2004; Parrott and Blunt 2005). Although experimental design variables make some of the endpoint sensitivity comparisons challenging, it does not appear that there are substantial differences in EE2 test concentrations that produce adverse effects on egg production/fertility, versus those that alter the mechanism-specific endpoints. Hence from a pragmatic perspective, at least for the near-term, it seems reasonable to base an EE2 ALC on traditional measures of long-term reproductive success in fish.

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APPENDIX A

Table A.1. Effects of EE2 on Aquatic Animals (Short-term survival).

Species	Life stage	Method	Duration	EC50 or LC50 (ng/L)	Reference	Remarks
Traditional Acute (1-7 day timeframe)						
*Sea urchin, *Strongylocentrotus purpuratus*	embryo	S,U	96 h	30,000	Roepke 2005	EC50 - abnormal development
*Sea urchin, *Lytechinus anamesus*	embryo	S,U	96 h	30,000	Roepke 2005	EC50 - abnormal development
*Copepod, *Acartia tonsa*	egg	R,U	5 d	88,000	Andersen et al. 2001	EC50-Inhibition of naupliar development
Medaka, *Oryzias latipes*	adult	-	96 h	>1,000,000	Thompson 2000	
*Copepod, *Acartia tonsa*	10-12 d adult	S,U	48 h	1,100,000	Andersen et al. 2001	
*Opossum shrimp, *Neomysis integer*	Juv, 2-4 mm	R, U	96 h	1,200,000	Verslycke et al. 2004	
Zebrafish, *Danio rerio*	adult	-	96 h	1,700,000	Wenzel et al. 2001	
Cladoceran, *Ceriodaphnia reticulata*		S,U	24 h	1,800,000	Jaser et al. 2003	EC50 mobility
Cnidarian, *Hydra vulgaris*	Adult male	R, U	96 h	3,800,000	Pascoe et al. 2002	
Cladoceran, *Sida crystallina*		S,U	24 h	>4,100,000	Jaser et al. 2003	EC50 mobility
Cladoceran, *Daphnia magna*	<24 h	S,U	48 h	>5,000,000	Goto and Hiromi 2003	
Midge, *Chironomus riparius*	4th instar	S,M	24 h	9,100,000	Lee and Choi 2006	

*Indicates saltwater species.

Table A.2. Effects of EE2 on Aquatic Animals (Long-term survival).

Species	Life Stage	Method	Duration	NOEC-survival (ng/L)	LOEC - survival (ng/L)	Reference	Remarks
Significant Effect Observed							
Zebrafish, *Danio rerio*	1 d old	R,U	38 d	10	100	Orn et al 2006	100% mortality at LOEC
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	10	100	Hill and Janz 2003	90% mortality at LOEC (45% control mortality); excess solvent
Medaka, *Oryzias latipes*	1 d	R,M	LC: 85 to 110 dph	29	290	Metcalfe et al. 2001	83% mortality at LOEC
*Sheepshead minnow, *Cyprinodon variegatus*	juv	F,M	PLC:59-7 dph F1	120	330	Zillioux et al. 2001	50% mortality at LOEC (42 days)
Medaka, *Oryzias latipes*	6 mo.	F,M	21 d	260	490	Seki et al. 2002	42% mortality at LOEC (4 of 5 dead males)
Rainbow trout, *Oncorhynchus mykiss*	1+ year	F,M	62 d pre-spawning	130	750	Schultz et al. 2003	100% mortality at LOEC (57 days)
Medaka, *Oryzias latipes*	4 mo.	R,U	14 d	500	2,000	Thompson 2000; Tilton et al. 2005	Significant mortality at LOEC
Zebrafish, *Danio rerio*	fert. eggs	R,U	5 wk	-	5,000	Ortiz-Zarragoitia et al. 2006	50% mortalty of exposed animals
*Copepod, *Tisbe battagliai*	<24 h	R,U	10 d	-	>100,000	Hutchinson et al. 1999	Value is an LC50
Wood frog, *Rana sylvatica*	Gosner 26	R,U	14 d	-	560,000	Hogan et al. 2006	Value is an LC50
Amphipod, *Gammarus pulex*	3-5 mm	R,M	10 d	-	840,000	Watts et al. 2001	Value is an LC50
Leopard frog, *Rana pipiens*	Gosner 26	R,U	14 d	-	890,000	Hogan et al. 2006	Value is an LC50
Leopard frog, *Rana pipiens*	Gosner 36	R,U	14 d	-	1,200,000	Hogan et al. 2006	Value is an LC50
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)							
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	6	-	Robinson et al. 2003	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	10	-	Parrot et al. 2003	
Fathead minnow, *Pimephales promelas*	juvenile	F,M	21 d	20	-	Panter et al. 2002	Conc. only 40% of nominal

Species	Life Stage	Method	Duration	NOEC-survival (ng/L)	LOEC - survival (ng/L)	Reference	Remarks
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	25	-	Zha et al. 2007	10% mortality
Zebrafish, *Danio rerio*	fert. eggs	F,U	3 mo	25	-	Van den Belt et al. 2003	40% mortality after 5 mo. recovery
Fathead minnow, *Pimephales promelas*	mature male	F,U	35 d	50	-	Schmid et al. 2002	
Snail, *Potamopyrgus antipodarum*	adult	R,U	9 wk	100	-	Jobling et al. 2004	
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	100	-	Scholz and Gutzeit 2000	8% mortality
Zebrafish, *Danio rerio*	4 wk	R,U	33 d	100	-	Versonnen and Janssen 2004	6.6% mortality; excessive carrier solvent
Guppy, *Poecilia reticulate* (male)	< 7 d	F,M	108 d	110	-	Nielsen and Baatrup 2006	
Sturgeon, *Acipenser fulvescens*	1 yr	F,M	25 d	120	-	Palace et al. 2001	
Rainbow trout male, *Oncorhynchus mykiss*		F,M	3 wk	130	-	Hook et al. 2007	
African clawed frog, *Xenopus laevis*	adult	R,U	4 wk	2,960	-	Urbatzka et al. 2007	
Wood frog, *Rana sylvatica*	Gosner stage 25	R,M	76 d	4,100	-	Mackenzie et al. 2003	
Amphipod *Gammarus pulex*	mixed age	F,M	100 d	7,600	-	Watts et al. 2002	
Amphipod adult, *Hyalella azteca*	pre-copulatory	R,M	10 wk - 2 x gen	10,000	-	Vandenbergh et al. 2003	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 d	100,000	-	Hutchinson et al. 1999	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 d	100,000	-	Pounds et al. 2002	
Cladoceran, *Sida crystallina*		R,U	34 d	500,000	-	Jaser et al. 2003	
Cladoceran, *Daphnia magna*		R,U	25 d	500,000	-	Goto and Hiromi 2003	

*Indicates saltwater species.

Table A.3. Effects of EE2 on Aquatic Animals (Growth).

Species Significant Effects Observed	Life Stage	Method	Duration	NOEC-growth (ng/L)	LOEC-growth (ng/L)	Reference	Remarks
Zebrafish , *Danio rerio*	fert egg	F,M	LC – F1	0.10	0.3	Wenzel et al. 2001	7% reduction at LOEC (75 dph)
Zebrafish , *Danio rerio*	fert egg	F,M	PLC	0.30	1.1	Wenzel et al. 2001	2% reduction at LOEC (78 dph)
Zebrafish, *Danio rerio*	20 dph	R,M	40 d	0.60	1.5	Orn et al. 2003	**Increased** juvenile wet weight
Fathead minnow, *Pimephales promelas*	<24 hr	F,M	LC	0.76	2.8	Länge et al. 2001	
Zebrafish , *Danio rerio*	fert egg	F,U	3 mo	1	10	Van den Belt et al. 2003	
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	1	10	Lin and Janz 2006	
Fathead minnow, *Pimephales promelas*	embryo	F,M	114 d	-	12	Bogers et al. 2006b	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 days	5	25	Zha et al. 2007	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	10	32	Parrot and Wood 2002	
Zebrafish , *Danio rerio*	4 wk	R,U	33 d	10	100	Versonnen and Janssen 2004	Excessive carrier solvent
Guppy, *Poecilia reticulata*	<7d	F,M	108 d	44	112	Nielsen and Baatrup 2006	**Increased** adult wet weight.
Medaka, *Oryzias latipes*	1 day old	R,M	LC	29	290	Metcalfe et al. 2001	
Midge, *Chironomus riparius*	4th instar	S,U	48 hr	50	500	Lee et al. 2006	I**ncreased** larval dry weight
Midge, *Chironomus riparius*	1st instar	R,M	egg - pupa	100,000	1,000,000	Watts et al. 2003	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)							
Zebrafish, *Danio rerio*	fert egg	F,M	2xGen	4.5	-	Nash et al. 2004	
*Three-spined stickleback, *Gasterosteus aculeatus*	fry	R,U	14 days	7.3	-	Hahlbeck et al. 2004b	

Species	Life Stage	Method	Duration	NOEC-growth (ng/L)	LOEC-growth (ng/L)	Reference	Remarks
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	10	-	Parrot et al. 2003	
Fathead minnow, *Pimephales promelas*	juv	F,M	21 days	20	-	Panter et al. 2002	
Prosobranch mollusc, *Potamopyrgus antipodarum*	adult	R,U	9 wk	100	-	Jobling et al. 2004	
*Sheepshead minnow , *Cyprinodon variegatus*	juv	F,M	PLC	330	-	Zillioux et al. 2001	
Wood frog , *Rana sylvatica*	Gosner stage 25	R,M	76 d	4,100	-	Mackenzie et al. 2003	

*Indicates saltwater species.

Table A.4. Chronic Reproductive Effects of EE2 on Aquatic Animals (Fecundity, Fertility, and Population Growth).

Species	Life Stage	Method	Duration	Endpoint	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Significant Effects Observed								
Fathead minnow, *Pimephales promelas*	40-60 h	F,M	LC	Percent fertilized of eggs laid	-	0.32	Parrott and Blunt 2005	
Zebrafish, *Danio rerio*	fert egg	F,M	LC	Number of fertilized eggs per female	0.30	1.1	Wenzel et al. 2001	
Fathead minnow, *Pimephales promelas*	<24 hr	F,M	LC	Mean no. eggs laid per breeding day	0.76	2.8	Länge et al. 2001	
Zebrafish, *Danio rerio*	fert egg	F,M	118 d	No. eggs spawned and prop fertilized	-	3	Fenske et al. 2005	
Fathead minnows, *Pimephales promelas*	-	Field	3 yrs	Population crash	-	3.2 – 8.9	Kidd et al. 2007	
Green frog, *Rana clamitans*	fert egg	Field	2 yr	Hatching success	-	3.2 – 8.9	Park and Kidd 2005	
Zebrafish, *Danio rerio*	fert egg	F,M	2 x gen	Proportion of non-viable eggs	0.50	4.5	Nash et al. 2004	Complete Rep. failure at LOEC
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 months	Fertile eggs and hatching success	-	6	Robinson et al. 2003	
Fathead minnow, *Pimephales promelas*	6-11 mo.	F,M	3 wk	Fert rate and no. eggs spawned	0.75	7.5	Pawlowski et al. 2004	**Increased** No. eggs spawned up to 0.75 ng/L
Zebrafish, *Danio rerio*	2 dph	R,U	60 d	% viable eggs, % hatch, % swim-up	1	10	Hill and Janz 2003	Excessive carrier solvent
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	Female egg production	1	10	Scholz and Gutzeit 2000	No effect on male fert at 10 ng/L
Zebrafish, *Danio rerio*	fert egg	F,U	3 mo	No. spawning females & egg prod	1	10	Van den Belt et al. 2003	
Zebrafish, *Danio rerio*	8 mo	R,U	14 d	Absence of intact eggs in ovaries	1	10	Versonnen and Janssen 2004	
Rainbow trout, *Oncorhynchus mykiss*	1+ year	F,M	PLC	Fertilization success	-	16	Schultz et al. 2003	EC50; same response@131 ng/L
Snail, *Potamopyrgus antipodarum*	adult	R,U	9 wk	Embryo production	25	100	Jobling et al. 2004	EE2 at 25 ng/L stimulatory
*Sheepshead minnow, *Cyprinodon variegatus*	juv	F,M	PLC	Hatching success	18	120	Zillioux et al. 2001	
Medaka, *Oryzias latipes*	6 mo.	F,M	21 d	Fecundity	260	490	Seki et al. 2002	

Species	Life Stage	Method	Duration	Endpoint	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Medaka, *Oryzias latipes*	4 mo.	R,U	14 d	Spawning frequency, % fertilized and % hatch.	5	500	Thompson 2000; Tilton et al. 2005	
Cladoceran, *Daphnia magna*	-	R,U	25 d	Embryo production	20,000	100,000	Goto and Hiromi 2003	
Rotifer, *Brachionus calyciflorus*		S,U	72 hr	Ratio of ovigerous/non-ovigerous females	202,000	510,000	Radix et al. 2002	
Rotifer, *Brachionus calyciflorus*		S,U	72 hr	Intrinsic rate population increase r	510,000	1,300,000	Radix et al. 2002	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Fathead minnow, *Pimephales promelas*	>6 mo.	F,M	3 wk	No. spawnings and eggs per spawn	1.5	-	Brian et al. 2007	
Mink frog, *Rana septentrionalis*	fert egg	Field	2 yr	Hatching success	3.2 – 8.9	-	Park and Kidd 2005	
Zebrafish, *Danio rerio* (females)	5-6 mo	R,U	15 d	Sterility in females	5,000	-	Ortiz-Zarragoitia et al. 2006	
Amphipod, *Gammarus pulex*	mixed ages	F, M	100 d	Population growth (total pop. size)	7,600	-	Watts et al. 2002	**Increase** in population size
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 days	Fecundity	100,000	-	Hutchinson et al. 1999	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 days	Reproduction	100,000	-	Pounds et al. 2002	

*Indicates saltwater species.

Table A.5. Chronic Reproductive Effects of EE2 on Aquatic Animals (Sex Reversal).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Significant Effects Observed								
Zebrafish, *Danio rerio*	fert egg	F,U	3 mo	Delayed sexual differentiation	-	0.10	Van den Belt et al. 2003	No males at LOEC
Zebrafish, *Danio rerio*	20 dph	R,M	40 d	Male:female sex ratios	-	0.60	Orn et al. 2003	Complete sex reversal at 1.5 ng/L
Fathead minnow, *Pimephales promelas*	40-60 h old	F,M	LC	Male:femal sex ratio	0.32	0.96	Parrott and Blunt 2005	Complete ex. femin. at 3.5 ng/L
Fathead minnow, *Pimephales promelas*	fert. eggs	F,U	60 d	Male:female sex ratio	0.32	1.0	Parrot and Wood 2002	Complete ex. femin. at 3.2 ng/L
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Male:female sex ratio	-	1.0	Lin and Janz 2006	
Fathead minnow, *Pimephales promelas*	<24 hr	F,M	LC	Sex reversal - all female	0.76	2.8	Länge et al. 2001	Sex ratio at 0.76 ng/L 54:46
Zebrafish, *Danio rerio*	fert egg	F,M	42 d	Male feminization	-	3.0	Fenske et al. 2005	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	Sex reversal - all female	-	10	Parrot et al. 2003	
Zebrafish, *Danio rerio*	1 dph	R,U	60 d	Complete feminization	-	10	Orn et al 2006	
Zebrafish, *Danio rerio*	1 dph	R,U	60 d	Complete feminization	-	10	Yamani 2004	
Fathead minnow, *Pimephales promelas*	embryo	F,M	114 d	75% female gonads; 15% un-developed	-	12	Bogers et al. 2006b	
Zebrafish, *Danio rerio*	fert egg	R,M	60 d	Complete feminization	-	15	Andersen et al. 2003b	
Medaka, *Oryzias latipes*	1 d	R,M	LC	Male:female sex ratio	2.9	29	Metcalfe et al. 2001	
*Three-spined stickleback, *Gasterosteus aculeatus*	Larvae	R,U	42 d	Sex reversal and intersex	-	50	Hahlbeck et al. 2004a	
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	Sex reversal with ovary	10	100	Scholz and Gutzeit 2000	
Medaka, *Oryzias latipes*	1 d	R,U	60 d	88% female, 2% male, 10% intersex	10	100	Yamani 2004 and Orn et al 2006	
Amphipod, *Gammarus pulex*	mixed ages	F, M	100 d	Male:femal ratio	-	104	Watts et al. 2002	No dose-response >104 ng/L

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Guppy, *Poecilia reticulate*	< 7 d	F,M	108 d	Male:female sex ratio	44	110	Nielsen and Baatrup 2006	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Green frog, *Rana clamitans*	fert egg	Field	2 yr	Male:female sex ratio	3.2 – 8.9	-	Park and Kidd 2005	
Mink frog, *Rana septentrionalis*	fert egg	Field	2 yr	Male:female sex ratio	3.2 – 8.9	-	Park and Kidd 2005	
Amphipod, *Hyalella azteca*	adult	R,M	10 wk; 2 gen	Male:femal sex ratio	10,000	-	Vandenbergh et al. 2003	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 d	Male:female sex ratio	100,000	-	Hutchinson et al. 1999	
Cladoceran, *Daphnia magna*	-	R,U	25 d	Male:female ratio	500,000	-	Goto and Hiromi 2003	

*Indicates saltwater species.

Table A.6. Chronic Reproductive Effects of EE2 on Aquatic Animals (Intersex).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Significant Effects Observed								
Pearl dace, *Margariscus margarita*	mature	Field	3 yr	Presence of testis-ova	-	3.2 – 8.9	Palace et al. 2006	Edema in ovaries
Fathead minnows, *Pimephales promelas*		Field	3 yr	Presence of testis-ova	-	3.2 – 8.9	Kidd et al. 2007	Testicular malformations
Mink frog, *Rana septentrionalis*	fert eggs	Field	2 yr	Intersex gonads (5 – 12 %)	-	3.2 – 8.9	Park and Kidd 2005	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Testis-ova in males	1.0	5.0	Zha et al. 2007	No sperm detectable
Fathead minnow, *Pimephales promelas*	egg	F,U	5 d	Ovarian cavities in males (8%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	5-10 dph	F,U	5 d	Ovarian cavities in males (38%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	10-15 dph	F,U	5 d	Ovarian cavities in males (64%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	15-20 dph	F,U	5 d	Ovarian cavities in males (43%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	egg	F,U	20 d	Ovarian cavities in males (22%)	-	10	Van Aerle et al. 2002	
Medaka, *Oryzias latipes*	1 d	R,M	LC	Sex inversion and testis-ova	2.9	29	Metcalfe et al. 2001	4 of 4 males with TO
*Three-spined stickleback, *Gasterosteus aculeatus*	Larvae	R,U	42 d	Intersexed gonads	-	50	Hahlbeck et al. 2004a	
Medaka, *Oryzias latipes*	6 mo	F,M	21 d	Testis-ova in males (33%)	33	64	Seki et al. 2002	No histological abnormalities in females
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	All males developed an ovary	10	100	Scholz and Gutzeit 2000	No effect on male fertility
Medaka, *Oryzias latipes*	1 d	R,U	60 d	Intersexed gonads (10%)	10	100	Yamani 2004 and Orn et al 2006	
Guppy, *Poecilia reticulata*	< 7 d	F,M	108 d	Feminization of male reproductive ducts	44	110	Nielsen and Baatrup 2006	
Amphipod, *Hyalella azteca*	fert eggs	R,M	2 x gen	Oocyte-like structures in males	23	320	Vandenbergh et al. 2003	
Leopard frog, *Rana pipiens*	Gosner 25	R,M	162 d	Intersex and altered testicular develepment	414	4,140	Mackenzie et al. 2003	

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Wood frog, *Rana sylvatica*	Gosner 25-28	R,M	76 d	Intersex and altered testicular development	-	4,140	Mackenzie et al. 2003	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Green frog, *Rana clamitans*	fert eggs	Field	2 yr	Intersexed gonads	3.2 – 8.9	-	Park and Kidd 2005	
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Testis-ova	10	-	Lin and Janz 2006	
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Testis-ova	10	-	Hill and Janz 2003	Excessive carrier solvent; high control mortality
Zebrafish, *Danio rerio*	adult	F,U	21 d	Feminization of testes	25	-	Islinger et al. 2003	

*Indicates saltwater species.

Table A.7. Chronic Reproductive Effects of EE2 on Aquatic Animals (Sexual Behavior).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Significant Effects Observed								
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	Male nesting behavior	-	6	Robinson et al. 2003	High mortality in solvent controls in first month of exposure
Fathead minnow, *Pimephales promelas*	mature	F,M	27 d	Impaired ability to compete and acquire territory	2.0	8.9	Majewski et al. 2002	
*Three-spined stickleback, *Gasterosterus aculeatus*	mature	R,U	12 d	Time spent near nest and glueing frequency, but effect short-lived	-	10	Brian et al. 2006	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Zebrafish, *Danio rerio*	fert egg	F,M	2 x gen	Natural spawning behavior of adult male fish	4.5	-	Nash et al. 2004	Sexually compromised males still actively participated in the spawning act, i.e., chasing females and competing with healthy males
Amphipod, *Gammarus pulex*	3-5 mm	R,M	10 d	Pre-copulatory guarding behavior	3,700,000	-	Watts et al. 2001	Reproductive behavior was only disrupted at high concentrations where it would be unrealistic to attribute effects to and endocrine-mediated process.

Table A.8. Data on Effects of EE2 on Aquatic Animals (Secondary Sexual Characterstics).

Species	Life Stage	Method	Duration	Effect	Event Association	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Significant Effects Observed									
Fathead minnow, *Pimephales promelas*	6-11 mo.	F,M	3 wk	Number of male nuptial tubercles	Activational	-	0.80	Pawlowski et al. 2004	
Fathead minnow, *Pimephales promelas*	40-60 h	F,M	LC	Ovipositor size, nuptial tubercles, banding strength	Organizational	0.32	0.96	Parrott and Blunt 2005	
Fathead minnow, *Pimephales promelas*	156 dph	F,M	LC	Nuptial tubercles, banding strength, dorsal fin dot, dorsal fat pad	Activational	0.32	0.96	Parrott and Blunt 2005	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	Male sex index: nuptial tubercles, dorsal fat pad, dorsal fin dot, banding strength	Organizational	0.32	1.0	Parrot and Wood 2002	Complete femin. at 3.2 ng/L
Fathead minnow), *Pimephales promelas*	<24 hr	F,M	LC	Secondary sex characteristics – not specified	Organizational	0.76	2.8	Länge et al. 2001	50% sex ratio at 0.76 ng/L
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	Development and length of ovipositors	Organizational	1.0	3.2	Parrot and Wood 2002	Complete femin. at 3.5 ng/L
Zebrafish, *Danio rerio*	fert egg	F,M	2 x gen	Coloration and bright anal fin markings	Organizational	-	4.5	Nash et al. 2004	
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	Delayed and inhibited nuptial coloration in males	Activational	-	6.0	Robinson et al. 2003	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	Ovipositor size, nuptial tubercles, banding strength	Organizational	-	10	Parrot et al. 2003	
Fathead minnow, *Pimephales promelas*	maturing	F,M	21 d	Number and prominence of nuptial tubercles and dorsal fat pad	Activational	-	11	Filby et al. 2007	
Fathead minnow, *Pimephales promelas*	embryo	F,M	114 d	Number and prominence of nuptial tubercles	Organizational	-	12	Bogers et al. 2006b	
Amphipod, *Hyalella azteca*	fert eggs	R,M	2 x gen	Male second gnathopods	Organizational	-	23	Vandenbergh et al. 2003	No effect >1,000 ng/L
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)									
Fathead minnow, *Pimephales promelas*	>6 mo.	F,M	3 wk	Relative fat pad weight, number of nuptial tubercles, nuptial tubercle prominence	Activational	1.5	-	Brian et al. 2007	
Medaka, *Oryzias latipes*	4 mo	R,U	14 d	Anal and dorsal fin shape	Organizational	500	-	Thompson 2000; Tilton et al. 2005	

*Indicates saltwater species.

Table A.9. Chronic Reproductive Effects of EE2 on Aquatic Animals (Vitellogenin).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Significant Effects Observed								
Zebrafish, *Danio rerio*	adult males	F,M	40 d	Increased whole-blood VTG level	-	0.50	Nash et al. 2004	
Fathead minnow, *Pimephales promelas*	6-11 mo	F,M	3 wk	Increased plasma VTG levels	-	0.80	Pawlowski et al. 2004	
Rainbow trout, *Oncorhynchus mykiss*	immature female	F,M	14 d	Increased liver *vtg* mRNA expression and plasma VTG	0.21	1.0	Thomas-Jones et al. 2003	
Zebrafish, *Danio rerio*	20 dph	R,M	40 d	Increased whole body VTG levels	-	1.5	Orn et al. 2003	
Zebrafish ,*Danio rerio*	adult male	R,U	21 d	Increased plasma VTG level	-	1.6	Fenske et al. 2001	
Rainbow trout, *Oncorhynchus mykiss*	Adult male	F,M	3 wk	Increased plasma VTG level	-	1.8	Jobling et al. 1996	
Zebrafish, *Danio rerio*	fert egg	F,M	42 d	Increased vitellogenin level	-	3.0	Fenske et al. 2005	
Fathead minnow, *Pimephales promelas*	-	Field	5 mo	Increased plasma VTG levels	-	3.2 – 8.9	Palace et al. 2002 (also see Kidd et al. 2007)	
Pearl dace, *Margariscus margarita*	-	Field	3 yrs	Increased whole body VTG level	-	3.2 – 8.9	Palace et al. 2006	
Zebrafish, *Danio rerio*	adult male	F,M	8 d	Increased whole-body VTG level	2.2	3.6	Rose et al. 2002	EC10 = 0.92 ng/L
Zebrafish, *Danio rerio*	adult females	F,M	40 d	Increased whole-blood VTG level	0.50	4.5	Nash et al. 2004	
Fathead minnow, *Pimephales promelas*	juv	F,M	21 d	Increased whole-body VTG level	2.0	5.0	Panter et al. 2002	
Fathead minnow, *Pimephales promelas*	8 mo	R,M	48 hrs	Increased liver *vtg* levels	2.5	5.0	Biales et al. 2007	
Ide, *Leucisus idus*	juv	F,M	7 d	Increased plasma VTG levels	-	6.0	Allner et al. 1999	
Zebrafish, *Danio rerio*	fert egg	R,M	4 d	Increased whole body VTG level	2.6	7.8	Bogers et al. 2006a	
Zebrafish, *Danio rerio*	adult females	R,M	21 d	Increased plasma VTG level	4.1	8.5	Van den Belt et al. 2004	
Zebrafish, *Danio rerio*	adult males	F,M	24 d	Increased plasma VTG level	-	9.0	Van den Belt et al. 2002	

Rainbow trout, *Oncorhynchus mykiss*	11 mo	F,M	2 wk	Increased plasm VTG level	0.87	10	Samuelsson et al. 2006	
*Eelpout, *Zoarces viviparus*	adult female	F,M	3 wk	Increased plasma VTG level	5.0	10	Korsgaard et al. 2002	
Fathead minnow), *Pimephales promelas*	<24 hr	F,M	LC	Increased whole body VTG level	2.8	12	Länge et al. 2001	
Zebrafish, *Danio rerio*	adults	F,M	168 hrs	Increased plasma VTG level	-	14	Hoffmann et al. 2006	
Sturgeon, *Acipenser fulvescens*	1 yr	F,M	25 d	Increased plasma VTG levels	-	14	Palace et al. 2001	
Lake trout, *Salvelinus namaycush*	immature	F,M	21 d	Increased plasma VTG level	-	15	Werner et al. 2003	Excessive carrier solvent
*Baltic flounder, *Platichthys flesus*	adult	F,M	21 d	Increased plasma VTG level in male and female fish	-	15	Allen et al. 1999b	
Medaka, *Oryzias latipes*	6 mo.	F,M	21 d	Increased liver Vtg levels	33	64	Seki et al. 2002	
Rainbow trout, *Oncorhynchus mykiss*	juvenile	R,M	14 d	Increased plasma VTG level	10	100	Verslycke et al. 2002	
*Sheepshead minnow, *Cyprinodon variegatus*	male	F,M	16 d	Increased liver *vtg* mRNA expression	24	110	Folmar et al. 2000	
Rainbow trout, *Oncorhynchus mykis*s	mature male	F,M	61 d	Increased plasma VTG levels	-	140	Skillman et al. 2006	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Zebrafish, *Danio rerio*	adult males	F,M	310 dpf F1	No effect on whole-blood VTG level	4.5	-	Nash et al. 2004	
Zebrafish, *Danio rerio*	adult females	F,M	310 dpf F1	No effect on whole-blood VTG level	4.5	-	Nash et al. 2004	

*Indicates saltwater species.

Table A.10. Chronic Effects of EE2 on Aquatic Animals (Other Relevant Endpoints).

Species	Life Stage	Method	Duration	Effect	Concentration (ng/L)	Reference	Remarks
Significant Effects Observed							
Zebrafish , *Danio rerio*	17-20 dpf	R,U	3 d	Enhanced effect on CYP19A2 gene expression	0.30	Kazeto et al. 2004	Excessive carrier solvent
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Suppression of gametogenesis for males (no testes discernable) and females	1.0	Weber et al 2003	Excessive carrier solvent
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Increased GSI and renal somatic index (RSI) in males	1.0	Zha et al. 2007	
Fathead minnow, *Pimephales promelas*	40-60 h	F,M	LC	Reduced GSI in females	3.5	Parrott and Blunt 2005	
Atlantic salmon, *Salmo salar*	immature	S,U	7 d	Increased AchE and GST activities and lactate content after 3 days, but no effect at 7 days	5.0	Greco et al. 2007	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Reduced GSI in females	5.0	Zha et al. 2007	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Increased RSI in females	5.0	Zha et al. 2007	
Medaka, *Oryzias latipes*	4 mo	R,U	14 d	Increased male and female plasma E2 levels	5.0	Thomposn 2000; Tilton et al. 2005	
Fathead minnow, *Pimephales promelas*	6-11 mo	F,M	3 wk	Reduction on male GSI	7.5	Pawlowski et al. 2004	
Zebrafish, *Danio rerio*	Adult female	R,M	21 d	Reduced female ovarian somatic index	8.5	Van den Belt et al. 2004	
Rainbow trout, *Oncorhynchus mykiss*	11 mo	F,M	2 wk	Higher hepatosomatic index (HSI)	10	Samuelsson et al. 2006	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	Increased liver somatic index	10	Parrot et al. 2003	
Medaka, *Oryzias latipes*	1 d	R,U	4 mo	In both males and females, significantly increased number of necrotic hepatocytes and kidney tubule cells	10	Weber et al. 2004	
*Baltic flounder, *Platichthys flesus*	adult	F,M	21 d	Increased HSI in males	15	Allen et al. 1999	
Zebrafish, *Danio rerio*	adult male	S,M	21 d	Increased levels of cyp19a2 mRNA (aromatase)	21	Kallivretaki et al. 2006	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Increased HSI in males	25	Zha et al. 2007	

Species	Life Stage	Method	Duration	Effect	Concentration (ng/L)	Reference	Remarks
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Ovary degeneration in females	25	Zha et al. 2007	
Zebrafish, Danio rerio	adult male	F,M	7 d	Decreased testrosterone and 11-ketotestosterone levels	26	Andersen et al. 2006	
Atlantic salmon, *Salmo salar*	immature	S,U	72 hr	Induced expression of brain P450 Aromatase	50	Lyssimachou et al. 2006	
Sturgeon, *Acipenser fulvescens*	1 yr	F,M	25 d	Increased plasma Vit E, A1 and A2; Decreased Vit E and A in kidney	60	Palace et al. 2001	
*Sheepshead minnow, *Cyprinodon variegatus*	juv	F,M	PLC	Increased pathological condition of kidneys	120	Zillioux et al. 2001	Fish survived to reproduction
Rainbow trout, *Oncorhynchus mykiss*	mature male	F,M	3 wk	Changed gene expression profile	130	Hook et al. 2007	
Zebrafish , Danio rerio	18-21 d	R,U	72 hr	Stimulated expression of Cytochrome P450 aromatase (Aro-B)	300	Le Page et al. 2006	
Medaka, *Oryzias latipes*	mature	R,U	14 d	Induced ER protein and aromatase activity	500	Contractor et al. 2004	
Medaka, *Oryzias latipes*	adult	R,U	14 d	Increased hepatic estrogen receptor (ER)	500	Thompson 2000	
Medaka, *Oryzias latipes*	4 mo.	R,U	14 d	Decreased female and male GSI	500	Thompson 2000; Tilton et al. 2005	
African clawed frog, *Xenopus laevis*	adult	R,U	4 wk	Reduced Leutinizing hormone B mRNA expression	3,000	Urbatzka et al. 2006	
African clawed frog, Xenopus laevis	adult	R,U	4 wk	Reduced testosterone levels in both sexes	3,000	Urbatzka et al. 2007	
African clawed frog, Xenopus laevis	adult	R,U	4 wk	Reduced E2 level in females	3,000	Urbatzka et al. 2007	
Midge, *Chironomus riparius*	4th instar	S,U	24 h	Increased expression of heat shock proteins	8,000	Lee et al. 2006	
Medaka, *Oryzias latipes*	mature male	R,U	6 d	Increased mRNA expression of liver choriogenin L	10,000	Lee et al. 2002b	
Medaka, *Oryzias latipes*	mature male	R,U	6 d	Increased mRNA expression of liver choriogenin H levels	20,000	Lee et al. 2002b	
Medaka, *Oryzias latipes*	mature male	R,U	6 d	Increased mRNA expression of liver choriogenin H levels	20,000	Lee et al. 2002a	
Medaka, *Oryzias latipes*	juv	R,U	6 d	Increased mRNA expression of whole body Choriogenic H	50,000	Lee et al. 2002a	

No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)

Species	Life Stage	Method	Duration	Effect	Concentration (ng/L)	Reference	Remarks
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	No effect on GSI in males or females	6.0	Robinson et al. 2003	
Rainbow trout, *Oncorhynchus mykiss*	juvenile	R,M	14 d	No effect on GSI or HSI	100	Verslycke et al. 2002	
African clawed frog, *Xenopus laevis*	adult	R,U	4 wk	No effect on Gonadotropin Releasing Hormone mRNA expression	3,000	Urbatzka et al. 2006	

*Indicates saltwater species.