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DETAILED REVIEW PAPER ON AQUATIC ARTHROPODS IN LIFE CYCLE TOXICITY TESTS WITH AN EMPHASIS ON DEVELOPMENTAL, REPRODUCTIVE AND ENDOCRINE DISRUPTIVE EFFECTS

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No. 55

DETAILED REVIEW PAPER ON AQUATIC ARTHROPODS IN LIFE CYCLE TOXICITY TESTS WITH AN EMPHASIS ON DEVELOPMENTAL, REPRODUCTIVE AND ENDOCRINE DISRUPTIVE EFFECTS

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

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FOREWORD

This document presents a Detailed Review Paper (DRP) on Aquatic Arthropods in Life-Cycle Toxicity Tests with an Emphasis on Developmental, Reproductive and Endocrine Disrupting Effects. The initial document was prepared by the United States in 2002, to support the project on the development of a mysid two-generation test. Two commenting rounds were organized in 2002 and then in 2005. The scope of the document was extended to include more broadly aquatic arthropods species used in chronic tests including developmental and reproductive endpoints. Sweden agreed to take the lead for the finalization of the current document.

Comments submitted by national experts and BIAC and responses to comments were presented at the 17th Meeting of the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) in May 2005. The document was then finalized and approved at the 18th meeting of the WNT.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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PREAMBLE

In 1998, a Task Force on Endocrine Disrupter Testing and Assessment (EDTA) was established at the request of OECD member countries. The EDTA Task Force is a Special Activity of the Test Guidelines Programme and its main objectives are to:

- identify the needs and prioritize the development of new and enhanced guidelines for the detection and characterization of endocrine disrupting chemicals;
- develop a harmonized testing strategy for the screening and testing of endocrine disrupters;
- manage validation work for newly developed and enhanced Test Guidelines as appropriate; and,
- provide practical tools for sharing testing results and assessments.

The need for new and updated test methods to detect and characterize endocrine disrupting chemicals has been expressed by the Task Force for the assessment of human health effects and environmental effects. At early meetings of the EDTA Task Force, it appeared that existing OECD Test Guidelines would insufficiently cover for endocrine-related effects, especially for the environment. Member countries decided to list test methods which could potentially cover effects of chemicals on the reproductive system (estrogen agonists/antagonists and androgen agonists/antagonists) and on the development (thyroid system), and proposed enhancements where needed. Invertebrate life-cycle tests, encompassing development and reproduction, are proposed as *in vivo* assays including endpoints that indicate mechanisms of adverse effects (endocrine and other mechanisms) and potential population damage.

This Detailed Review Paper (DRP) is intended to provide the current state-of-the-knowledge in the area of aquatic invertebrate endocrinology, with a particular focus on the evaluation of possible developmental reproductive and endocrine disruptive effects in aquatic arthropods through life cycle tests.

The content of this review is intended to describe test methods, already existing or in development, that could be used in testing chemicals for their potential to interact with the endocrine system of aquatic arthropods and likely to create population damage. However, the methods described do not focus on endocrine disruption in terms of endpoints measured, but rather on reproductive and developmental performance of individuals exposed. It is planned that test methods under development or enhanced, will be validated to establish their relevance (i.e. whether the test is meaningful and useful for the intended purpose) and their reliability (i.e. reproducibility of test results over time within and among laboratories).

General principles for the conduct of validation studies have been defined following the OECD Stockholm Conference on Validation and Regulatory Acceptance of New and Updated Test Methods in Hazard Assessment. The OECD Guidance Document No. 34 describes these guiding principles and addresses the important steps and aspects that must be considered prior to and during the validation process. They include: (i) the definition of the test method and related issues (*e.g.,* purpose, decision criteria, endpoints, limitations); (ii) the design and conduct of pre-validation studies leading to the optimization of the test method; (iii) the design and conduct of the formal inter-laboratory validation work, based on the outcomes of the pre-validation studies and aiming at accumulation of data on the relevance and reliability of the test method, and; (iv) the overall data evaluation and subsequent validation study conclusion, keeping in mind the requirements of regulatory authorities, for submission of information relating to new or modified test procedures. It also discusses the need for and the extent of an independent evaluation, or peer review, of test methods being validated.

The planning and conduct of a validation study should be undertaken on a case-by-case basis since there may be several ways of assessing the validity of the method. As described in the Guidance Document No. 34, the validation process is sufficiently flexible so that it can be applied equally well to a wide variety of tests and procedures. The flexibility also applies regardless of whether tests are for health or environmental effects. Flexibility is also encouraged on issues such as the amounts of information required at each phase, the number of chemicals tested, when and to what extent to use blind testing, and the number of laboratories participating.

A Validation Management Group for Ecotoxicity Testing (VMG-eco) has been established at the OECD level to supervise the planning and conduct of experimental work in fish, birds, amphibians and invertebrates. This VMG-eco reports back to the Task Force on Endocrine Disrupters Testing and Assessment (EDTA). To discuss the technical details of the test methods, an *Ad hoc* Expert group on Invertebrate Testing was created in 2002 and met for the first time in October 2003 to discuss the various tests proposed and to prepare initial validation work.

The U.S. Environmental Protection Agency took the lead in preparing the initial and revised versions of this Detailed Review Paper for their national programme on endocrine disrupters. The draft document, initially called “DRP on a Mysid two-generation test”, was circulated for comments in 2002, discussed at the Second meeting of the Validation Management Group for Ecotoxicity tests (VMG-eco) and at the Seventh meeting of the Task Force on Endocrine Disrupters Testing and Assessment. These discussions and the comments received resulted in a recommendation to broaden the scope of the DRP to aquatic arthropods. The United States volunteered to revise the draft taking into account the recommendation and the initial comments compiled by the Secretariat in March 2003.

In December 2004, the United States submitted a revised version of the document, called “DRP on Aquatic Arthropods in life cycle and two-generation toxicity tests”. This DRP was circulated in January 2005 to members of the Invertebrate Expert Group and the VMG-eco for comments again. In the comments received as of 23 February 2005 experts acknowledged that aquatic arthropods are now taken into account in the review, but there is still a large proportion of the document dedicated to mysids. Additionally, some experts commented that this version of the DRP did not address all the publicly available literature on freshwater and marine arthropods that is relevant to endocrine disrupters.

At the 17th Meeting of the Working Group of National Coordinators of the Test Guidelines Programme (WNT) it was decided that aquatic arthropods should be better represented, including a thorough re-evaluation of the existing literature on the subject.

Sweden volunteered to take over the lead for the project to include contributions from experts, e.g. from Japan, Germany and the Netherlands, and thereby finalize the review. This was decided at the 17th WNT meeting. It was also decided that a very pragmatic approach should be taken in order to finalize the review for the next WNT meeting in May 2006. The work have taken place from late 2005 to early 2006 and responsible for the work have been experts at the Department of Applied Environmental Science (ITM) in Sweden, in co-operation with experts from e.g. the above mentioned countries, the US and the Swedish Chemicals Inspectorate (KemI).

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1.0 EXECUTIVE SUMMARY

i) The overall purpose of this Detailed Review Paper (DRP) is to provide state-of-the-art knowledge about (life-cycle) tests on aquatic arthropods. A special emphasis is put on developmental and reproductive toxicity as well as potential endocrine disruptive effects. To understand both the possibilities and the difficulties in identifying potential endocrine disrupting chemicals within future regulatory work, this DRP presents information on basic invertebrate endocrinology and gives an overview of endocrine disruption observed in these groups of species. In this context it is important to emphasize that endocrine disruptive- and other specific biological effects (e.g. enzymatic) related to chemical exposure will be covered by studying endpoints reflective of both developmental and reproductive impairment (i.e. full life-cycle studies), regardless if we know the specific mode-of-action or not. This is the strength with the life-cycle protocols presented in the following text. In addition to this, the DRP also presents a number of established and novel biochemical techniques and endpoints, which should be used in concert with the regular endpoints observed from the life-cycle testing in order to increase our understanding of modes-of-action.

ii) Invertebrates (especially arthropods such as insects and crustaceans) constitute the vast majority of animal species on earth. Although many invertebrate toxicity test protocols are routinely used in regulatory toxicity testing, few have been designed with endocrine-specific endpoints in mind. Although the growth, reproduction, development, and other aspects of invertebrate physiology and life cycle are known to be regulated by endocrine control, the endocrine systems and the hormones produced and used in the invertebrate body are not directly analogous to those of vertebrates. For example, ecdysone is a steroid hormone that regulates growth and molting in arthropods, and exhibits some functional and structural similarities to estrogen. Therefore, methods for testing effects of potential endocrine disrupters on aquatic arthropods are relevant to assess the adverse consequences of such chemicals on development and reproduction, through life-cycle or two-generation tests.

iii) This DRP reviews the endocrinology of aquatic invertebrates in general (annelids, mollusks, insects, crustaceans and echinoderms, mainly) and provides examples of endocrine disruption. Issues associated with extrapolation are also explained in **Section 3**.

iv) The remaining sections of the paper focus on aquatic arthropods. **Section 4** lists those species that have been studied most and where test methods have sometimes been developed to assess reproductive and developmental impairments following exposure to chemical substances. This is followed by an evaluation of possible endocrine disruption in aquatic arthropods.

v) A number of appropriate test species and standard protocols are presented in **Section 5**.

vi) In **Section 6** a description of endpoints reflective of developmental and reproductive impairment is presented.

vii) Responses observed following exposure to ecdysteroid (ant-)agonists, androgenic and estrogenic compounds and other hormonal disturbances are presented in **Section 7**.

viii) Research needs and implementation considerations are described in **Section 8**; followed by references in **Section 9**.

ix) In **Annex 1 to 4**, established or proposed OECD arthropod life cycle protocols are presented.

2.0 INTRODUCTION

2.1 PURPOSE OF AQUATIC ARTHROPODS LIFE-CYCLE AND TWO-GENERATION TOXICITY TESTS

1. Test methods proposed at Level 5 of the EDTA Conceptual Framework are meant *i)* to provide data on effects from endocrine and other mechanisms of action, and *ii)* to be used for assessment of adverse effects and potential population damage. To fulfill this purpose, tests are often longer-term studies designed to encompass critical life states and processes, a broad range of doses, and administration by relevant route of exposure. In addition, the effects associated with potential endocrine disrupters can be latent and not manifested until later in life or may not be apparent until reproductive processes occur in an organism's life history. Thus, tests for endocrine disruption often encompass two generations to address effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity.

2. The results from such long-term tests should be conclusive in documenting a discernable cause-and-effect relationship of chemical exposure to measurable manifestation in the test organisms. The tests are generally expected to:

- Establish exposure/concentrations/timing and effects relationships;
- Be sensitive;
- Assess relevant endpoints, i.e. indicative of developmental and reproductive impairment;
- Include a dose range for full characterization of effects;
- Adhere to good laboratory practices; and
- Be suitable for validation.

3. Invertebrates (especially arthropods such as insects and crustaceans) constitute the vast majority of animal species on earth. Although many invertebrate toxicity test protocols are routinely used in regulatory toxicity testing, few have been designed with endocrine-specific endpoints in mind. Although the growth, reproduction, development, and other aspects of invertebrate physiology and life cycle are known to be regulated by endocrine control, the endocrine systems and the hormones produced and used in the invertebrate body are not directly analogous to those of vertebrates. For example, ecdysone is a steroid hormone that regulates growth and molting in arthropods, and exhibits some functional and structural similarities to estrogen. It has been reported that the vertebrate androgen testosterone acts as an ecdysteroid antagonist in a crustacean (Mu and LeBlanc 2002a). Also, the aromatase inhibitor fenarimol, which prevents the conversion of testosterone to the vertebrate estrogen, has been demonstrated to inhibit ecdysteroid synthesis and interfere with normal molting processes in a crustacean (Mu and LeBlanc 2002b). However, other substances than vertebrate estrogens and other steroid hormones and mimics are likely to be more prone to interfere with the unique invertebrate hormone systems. Therefore, methods for testing arthropods for effects of potential endocrine disrupters are relevant to assess the adverse consequences of chemicals indicated to be endocrine active in screening and short-term assays and tests.

2.2 ACRONYMS AND ABBREVIATIONS

4. Table 1 lists the acronyms and abbreviations used in the detailed review paper (DRP), with the exception of commonly used units, such as h for hour or L for liter. Each of the acronyms and abbreviations is also introduced at first use in the text.

Table 1. Acronyms and abbreviations

20-E	20-hydroxyecdysone
4NP	4-nonylphenol
AFDW	Ash-free dry weight
ASTM	American Society for Testing and Materials
BpA	Bisphenol A
BPDH	Black pigment-dispersing hormone
CHH	Crustacean hyperglycemic hormone
CNS	Central nervous system
CYP	Cytochrome P450 enzyme
DAH	Dark adapting hormone
DDT	Dichlorodiphenyl trichloroethane
DES	Diethylstilbestrol
DRP	Detailed Review Paper
EC	Effect concentration
EC_{50}	Median effective concentration
EDC	Endocrine-disrupting chemical
EDSTAC	US EPA Endocrine Disrupters Screening and Testing Advisory Committee
EDSP	Endocrine Disrupters Screening Programme
EPA	Environmental Protection Agency
GIH	Gonad-inhibiting hormone
GSH	Gonad-stimulating hormone
GSS	Gonad-stimulating substance
HPV-inerts	High production volume inert compounds
IC	Inhibition concentration
IGR	Insect growth regulator
JH	Juvenile hormone
LAH	Light-adapting hormone
LC	Lethal concentration
LC_{50}	Median lethal concentration
LOEC	Lowest observed effects concentration
MAR	Metabolic androgenization ratio
MF	Methyl farnesoate

MIH	Molt-inhibiting hormone
MSD	Minimum significant difference
NIEHS	National Institute of Environmental Health Sciences
NOEC	No observed effect concentration
NPPE	Nonylphenol polyethoxylate
OECD	Organization for Economic Cooperation and Development
OPPTS	US EPA Office of Prevention, Pesticides and Toxic Substances
PCB	Polychlorinated biphenyl
PoA	Ponasterone A
SAB	Scientific Advisory Board
SAP	Scientific Advisory Panel
SDWA	Safe Drinking Water Act
TBT	Tributyltin

3.0 AQUATIC INVERTEBRATE ENDOCRINOLOGY AND ENDOCRINE DISRUPTION

5. Invertebrates comprise 95% of the world's animal species (Wilson 1988), and certainly a larger percentage of the Earth's total animal abundance. Certainly, it is understood that hormones are important in controlling physiological processes in invertebrates (Lafont 2000, Oehlmann and Schulte-Oehlmann 2003). However, until recently invertebrates received little attention regarding potential endocrine disruption, the main exception being studies of imposex in gastropods. Invertebrate endocrine systems, except perhaps those of arthropods, are generally poorly studied compared to those of vertebrates and this limits ability to evaluate potential EDCs (Lafont 2000, Oehlmann and Schulte-Oehlmann 2003). Because many invertebrate endocrine systems are not very well understood, some changes to endocrine systems after exposure to a chemical may not be detected or may not be measurable (Oehlmann and Schulte-Oehlmann 2003). However, the situation is improving with many studies being done within the last few years on such groups as sponges, corals, polychaetes, and echinoderms (Section 3.1), adding to what has been learned about arthropod endocrinology. Nonetheless, to gain a full understanding of the complex endocrine disruption issue, invertebrates must be included in a tiered testing approach (Vandenbergh et al. 2003).

3.1 OVERVIEW OF AQUATIC INVERTEBRATE ENDOCRINOLOGY

6. There are several recent general reviews that provide good summaries of invertebrate endocrinology (LeBlanc et al. 1999, Lafont 2000a, Feix and Hoch 2002). Additionally, there are some reviews that pertain to specific groups of invertebrates including Cnidaria (Leitz 2001), Annelida (Hardege 1999, Andries 2001, Salzet 2001), Insecta (e.g., Lafont 2000b), and Crustacea (Fingerman 1987, 1997, Huberman 2000, Subramoniam 2000). The intent of this section is to provide a brief overview of invertebrate endocrinology. More details can be found in the review articles.

7. As animal body plans increased in complexity from simple cell-based to organ system-based structures, the need for a means to coordinate internal processes became more important. Neural networks are developed in the Cnidaria, one of the more primitive invertebrate groups (Lafont 2000a). The complexity of the coordination systems increased through the invertebrate evolutionary line, progressing to the presence of endocrine cells in the Annelida, endocrine glands in the Mollusca, and perhaps culminating in complexity in the Arthropoda, although Lafont (2000a) offered that this conclusion might reflect the general lack of study of invertebrate endocrine systems. The complexity of coordinating systems is reflected in the pathways by which stimulation of the central nervous system (CNS) generates a response in a target organ (Lafont 2000a). The stimulus can induce the CNS to produce a neurotransmitter or a neurohormone. Both messengers can then act directly on the target organ or on an endocrine gland. The endocrine gland can then act on the target organ or on another endocrine gland, which then acts on the target organ.

8. There are two main types of chemical messengers (Lafont 2000a):

- Neurotransmitters may be fast- or slow-acting depending on whether the link to ion channels is direct or indirect (acetylcholine, glutamate); these are low molecular weight molecules and have low diversity throughout invertebrates, i.e., they are evolutionarily conservative.

- Neurohormones/hormones include peptide/protein and lipid molecules that can be grouped by similarity of structure (e.g., steroids, peptides, terpenoids); these messengers show important evolutionarily diversification and either are related to vertebrate messengers or are specific to invertebrates.

9. Lafont made two key generalizations about invertebrate endocrine systems that are important to consider when evaluating endocrine disruption in invertebrates: (1) there are two sets of hormones in invertebrates—those that are similar to those found in vertebrates (i.e., they likely share a common ancestry) and those specific to inverts (e.g., ecdysteroids); (2) structurally related molecules may have different functions. This last generalization is very important and has been shown to be applicable to some studies of EDCs (Section 3.2)

10. LeBlanc et al. (1999) gave an introductory overview of animal lineages as they relate to endocrinology. Briefly, animals diverged from the ancestral "stem" line into two primary lineages, the protostomes and the deuterostomes. Traditionally, embryological features have been used to separate the two lineages. Major stem groups that will be discussed here are the sponges (Porifera) and jellyfish, hydroids, and corals (all Cnidaria). Major Protostome groups include worms (Annelida), snails and clams (Mollusca), midges (Insecta), and amphipods, daphnids, copepods, mysids, and decapods (Crustacea). The primary deuterostomes mentioned here are sea stars and feather stars (Echinodermata) and fish (Vertebrata). This divergence into two main lineages corresponds to important endocrinological differences. Protostomes primarily rely on neuropeptides to regulate physiological processes, although more advanced groups (insects, crustaceans) have increased reliance on ecdysteroids and terpenoids. Invertebrate deuterostomes (echinoderms) rely more on vertebrate-like steroids (estrogens, androgens, progesterone) and terpenoids, but do not have ecdysteroid hormones.

11. LeBlanc et al. (1999) also discussed the endocrinology of the major invertebrate groups. Many studies of individual invertebrate groups have identified single hormones or groups of hormones. Examples of these studies, selected primarily to build on the LeBlanc et al. (1999) review, are discussed in the following sections. The groups are presented in approximate phylogenetic sequence. Oehlmann and Schulte-Oehlmann (2003) prepared a table of the hormones that have been identified for the major invertebrate taxa, and the processes they control. Table 2 is a modification of their table that has been updated to include some additional taxa and hormones that have been identified recently.

Table 2. Examples of hormone reported in Invertebrate Taxa. Modified from Summary table from Oehlmann and Schulte-Oehlmann (2003), unless indocated otherwise

Taxon	Hormone Type	Example	Controlled Process
Porifera	Unknown	Unknown	Unknown
Cnidaria	Neuropeptides Thyroids Retinoids Steroids	GLWamides Thyroxine 9-*cis*-retinoic acid 17β-estradiol [1]	Metamorphosis Strobilation Strobilation Reproduction
Nematoda	Ecdysteroids Terpenoids Neuropeptides	Unknown Juvenile hormone-like FMRFamide	Unknown Growth Unknown
Annelida	Ecdysteroids Neuropeptides Terpenoids	Ecdysone FMRFamide Gonadotropin [2] Eicosatrienoic acid [3] Aracidonic acid [3]	Unknown Neuromodulation Vitellogenesis Metamorphosis
Mollusca	Ecdysteroids Steroids Terpenoids Neuropeptides	Unknown Testosterone, 17β-estradiol, progesterone JH reported APGWamide, dorsal body hormone, FMRFamide, egg-laying hormone, molluscan insulin-like peptides	Unknown Sexual differentiation, prosobranch reproduction Questionable Sexual differentiation, gonad maturation, spawning, neuromodulation, growth, development, energy metabolism
Crustacea	Ecdysteroids Steroids Terpenoids Neuropeptides	Ecdysone 17β-estradiol, testosterone, progesterone Methyl farnesoate Androgenic hormone Crustacean Hyperglycemic Hormone Molt-inhibiting hormone Vitellogenesis-inhibiting hormone	Molting, vitellogenesis Uncertain Metamorphosis Sexual differentiation, vitellogenesis inhibition Energy metabolism Ecdysteroid production Vitellogenesis
Echinodermata	Steroids Neuropeptides	Testosterone, 17β-estradiol, estrone Gonad-stimulating substance Maturation-promoting factor	Vitellogenesis, gametogenesis, spawning Spawning Fertilization
Tunicata	Steroids Neuropeptides Thyroids	Testosterone. 17β-estradiol GRH analog Thyroxine	Gametogenesis, spawning Gonad development Tunic formation (?)

[1] Tarrant et al. (1999), Pernet and Anctil (2002). [2] Gaudron and Bentley (2002). [3] Laufer and Biggers (2001).

3.1.1 Porifera

12. The current literature review did not identify any studies that have demonstrated the occurrence of hormones or endocrine functions in the sponges. However, one study (Hill et al. 2002) did examine the effects of three chemicals known to disrupt endocrine systems in some animals on freshwater sponges (see Section 3.2.1).

3.1.2 Cnidaria

13. The cnidarians are of particular interest because of the group's position as one of the stem invertebrate phyla that existed before the divergence into the protostome and deuterostome lineages. Cnidarians may, therefore, show aspects of endocrinology conserved in both groups (LeBlanc et al. 1999). Also, some taxa within the phylum are of particular importance in tropical waters, e.g., coral reefs comprise a major ecosystem. Cnidarian neurosecretory cells may have been precursors to the evolution of the neurohormonal systems of higher animals (Feix and Hoch 2002).

14. Leitz (2001), in a thorough review of cnidarian endocrinology, reported that cnidarians do not possess defined endocrine glands, but that neurones are the major source of signaling compounds in cnidarians, although the target cells rarely have been identified. Leitz identified several major groups of regulatory compounds. Non-peptide regulatory compounds include catecholamines and their precursors (dopamine, adrenaline, norepinephrine), serotonin (5-hydroxytryptamine), taurine, and gamma-aminobutyric acid (GABA). The functions of most have not been identified clearly. Serotonin may be involved in metamorphosis, taurine may function in osmoregulation, and GABA may affect feeding.

15. Most of the peptide regulatory compounds are neuropeptides and inhibit or stimulate muscle contraction in hydrozoans and anthozoans (Leitz 2001). Gonadotropin-releasing hormones (GnRHs) comprise a peptide family that is conserved in length and amino-acid sequence composition (Anctil 2000). There is some evidence that they may also be active in other invertebrates (e.g., mollusks and tunicates). Anctil (2000) found evidence for GnRHs in the sea pansy *Renilla koellikeri* that are physiologically active and function in modulation of peristalsis (GnRH inhibits peristalsis), which is strongly enhanced during spawning. They were also found in starlet anemone *Nematostella vectensis*.

16. Retinoids occur in cnidarians. Retinoic acid X receptors (RXR) are nuclear hormone receptors found in vertebrates, echinoderms, arthropods, nematodes (Kostrouch et al. 1998). Kostrouch et al. found jellyfish RXR (jRXR), which is a close homolog of vertebrate RXR, in the jellyfish (*Tripedalia cystophora*). This hormone targets genes that encode soluble crystallins in lens of eye (as in vertebrates), which suggests that cnidarians are ancestral to other phyla, not an independent offshoot.

17. Sex steroids were identified in cnidarians very recently. Pernet and Anctil (2002) studied the sea pansy, which is dioecious and forms separate male and female colonies. Pernet and Anctil discovered the vertebrate estrogen 17-β estradiol (E_2) in all colonies and found that the levels varied through the reproductive cycle with a strong peak in March (at the onset of maturation) and June (start of the spawning period). These observations argue that 17-β estradiol has a role in the reproductive biology of the sea pansy. Tarrant et al. (1999) identified 17-β estradiol in a scleractinian coral (*Montipora capitata*) and thought that it functioned in reproduction. Corals contain a variety of steroids (Tarrant et al. 2003 among others), but there have been few studies on steroid metabolism. Water-born estrogens can be taken up by corals (Tarrant et al. 2001), metabolized (Tarrant et al. 2003), and can affect coral physiology (Tarrant et al. 2004). The presence of a vertebrate estrogen (17-β estradiol) in cnidarians is important for eventual understanding of evolution of hormonal systems.

3.1.3 Annelida

18. All hormones that have been identified in annelids to date are neuropeptides secreted by neurosecretory cells located primarily in the head (LeBlanc et al. 1999; Salzet 2001). A cardioactive peptide, FMRFamide (first found in mollusks), has been found in polychaetes, but not oligochaetes; ecdysteroids occur in some annelids, but no function for them has been determined; juvenile hormone affects larval settling when studied in the laboratory. Gaudron and Bentley (2002) discovered that the prostomium produces a gonadotrophic hormone that controls vitellogenesis in the oocytes. The hormone production is under environmental control. A second hormone induces oocyte maturation.

19. Andries (2001) reviewed the endocrine regulation of reproduction in polychaete worms. A single brain hormone controls reproductive development in worms that breed once and then die (semelparous reproduction) by inhibiting gonad maturation and promoting somatic growth. Hormone levels remain consistent throughout the extended period of gamete development and the population of oocytes, which includes various stages of development during the one- to three-year period, becomes homogeneous only when the hormone levels decrease. The hormone apparently also inhibits spermatogenesis. Pheromones are involved in the timing of broadcast spawning (Hardege 1999, Andries 2001). Reproduction in worms that breed annually or continuously (iteroparous reproduction) is regulated by a combination of environmental factors and hormones (e.g., supraesophogeal hormone stimulates ovarian-protein synthesis).

20. Laufer and Biggers (2001) reviewed the role of methyl farnesoate and juvenile hormone-active fatty acids in annelid metamorphosis and reproduction. They reported the *Capitella* trochophores respond very quickly to exposure to methyl farnesoate and eicosatrienoic acid (sperm maturation factor). Eicosatrienoic acid is present in adults of *Arenicola* and was proposed as a hormone that functions in metamorphosis and also induces spawning.

3.1.4 Mollusca

21. Neurosecretory centers, which produce neuropeptides, occur in the cerebral, pleural, pedal, abdominal ganglia of the central nervous system and comprise the molluscan endocrine system (LeBlanc et al. 1999). Typical invertebrate steroids (e.g., ecdysone) have been reported only rarely to occur in mollusks. Vertebrate-type steroids (testosterone, progesterone) can be synthesized from precursors in the ovotestes, which underscores the hermaphroditic character of many mollusks.

22. Ecdysteroids and juvenoids occur in some mollusks, but their particular functions have not been determined. The neuropeptide FMRFamide, a cardioaccelatory peptide that was first identified in mollusks, is one of the best known and most widespread neuroendocrine hormones. It regulates several physiological processes. Other peptides are involved in gonad maturation and egg production (egg-laying hormone), the development of female accessory sex organs, gonad maturation and ovulation (dorsal body hormones), and in growth, development, and metabolism (molluscan insulin-like peptides).

3.1.5 Insecta

23. Insect endocrinology, which is probably better understood than that of any other invertebrate group, has been thoroughly reviewed (e.g., LeBlanc et al. 1999, Lafont 2000b). Three types of structures comprise the endocrine system—neurosecretory cells, endocrine glands (epitracheal glands, corpora allata, prothoracic glands), and reproductive organs (LeBlanc et al. 1999). The neurosecretory cells produce neuropeptides that permit insects to respond to environmental factors that include food availability, temperature, and photoperiod, among others. Many hormones, most of which are unique to arthropods, have been identified in insects. Some insect hormones are similar to vertebrate hormones, but their functions have not been identified yet.

24. Two of the key insect hormones that are very relevant to an EDC evaluation program are ecdysteroids and juvenile hormones. Ecdysteroids, which are compounds structurally related to ecdysone (Goodwin et al. 1978), comprise one of the more important groups of insect hormones because they are involved in growth and development (molting) and reproduction (Lafont 2000b). Ecdysteroids are secreted by the prothoracic gland and by endocrine cells in the gonads. Insects cannot synthesize cholesterol; therefore, ecdysteroids are prepared from ingested cholesterol and plant steroids (LeBlanc et al. 1999). The synthetic pathway leading to the production of ecdysteroids is not completely known, partly because unstable compounds are included in some of its early stages (Lafont 2000b). Lafont mentioned two of the main questions concerning ecdysteroids that have yet to be answered: (a) How many active ecdysteroids and ecdysteroid receptors are there? and (b) How do varying concentrations of the hormones influence their action?

25. Juvenile hormones (JH) are terpenoids produced by the corpora allata that primarily regulate metamorphosis and reproduction. The presence or absence of JH determines the type of molt that occurs in the insect. The presence of JH during the initial rise of ecdysteroids in a larval stage will induce a molt to another larval stage (LeBlanc et al. 1999). When ecdysone levels start to rise during the final larval stage, the absence of JH will result in metamorphosis. In hemimetabolous species, this molt leads to the adult stage. In holometabolous species, JH again is present during this molt and the larva transforms into a pupa. The pupa molts to the adult in the absence of JH. JH biosynthetic pathways are known and are reviewed by Lafont (2000b). There are three main types of JH, called JHI, JHII, and JHIII, and several related compounds, including methyl farnesoate (MF) (Lafont 2000b). Lafont described a possible evolutionary scenario for JH with primitive insects (cockroaches) using MF and JHI and the most advanced insects (flies) using a unique form of JHIII (JHIII bisepoxide).

26. Many of the insecticides used to control outbreaks of agricultural pests have been formulated to interact with either of these two hormones. Many of these compounds, especially JH analogs, are not species-specific in action; thus they have the potential to impact non-target animals severely. Therefore, these compounds are often the focus of endocrine disruption studies.

3.1.6 Crustacea

27. For crustaceans, biological processes are regulated by a complex endocrine system (Cuzin-Roudy and Saleuddin 1989). Study of this regulation began about 1921 with the publication of the results of experiments by R. Courrier that showed that a hormone produced by an endocrine gland, not the testes, determined male secondary sex characteristics (Fingerman 1997). Fingerman's (1997) historical review traces the passage of crustacean endocrinology from its early development by emphasizing key discoveries such as the nature and role of the sinus gland and other glands, and the roles of specific hormones in crustacean biology. Many of the descriptions are enhanced by the author's personal experiences gained through 40 years of active research on crustacean endocrine systems. Crustacean endocrinology has been reviewed about every decade since the early studies of the 1920s and 1930s. Among the relatively recent reviews, Quackenbush (1986) reviewed studies of the four types of compounds that help regulate crustacean biology—peptides, steroids, terpenoids, and biogenic amines. Later reviews have approached crustacean endocrinology by examining physiological processes, particularly growth and reproduction (Charmantier et al. 1997, Chang 1997, Subramoniam 2000). Chang (1993) compared endocrine control of molting and reproduction in crustaceans to that in insects.

28. Basically, inputs from the environment are integrated by a central nervous system. Neurotransmitters and neuromodulators govern the release of neuropeptides, which govern the production of hormones by the endocrine glands (Cuzin-Roudy and Saleuddin 1989). Molting, for example, is controlled by the release of molting hormones, which are ecdysteroids, and by neurosecretions for the central nervous system, which are accumulated and released by the sinus gland. The main endocrine

centers for crustaceans described to date include the Y-organ, mandibular organ, androgenic gland, X-organ, and sinus gland.

29. Peptide hormones in crustaceans include compounds, such as red pigment concentrating hormone (RPCH) and pigment concentrating hormone, that affect the chromatophores and retinal pigments that comprise the complex color control systems found in crustaceans (Quackenbush 1986). Peptides also affect crustacean molting and reproduction. Molt-inhibiting hormone (MIH) is one factor that regulates molting. Peptides function in the control of vitellogenesis in crustaceans. Crustacean hyperglycemic hormone (CHH) is a peptide that regulates blood sugar, particularly glucose, in crustaceans (Quackenbush 1986). The hormone is unique among crustaceans in that it is taxon-specific. For example, CHH produced by crayfish does not affect crabs.

30. Steroid hormones include the ecdysteroids, which are the molting hormones in crustaceans (Charmantier et al. 1997, Chang 1997). These growth-regulating hormones also function in the control of reproduction and embryogenesis (Subramoniam 2000). Ecdysteroids are synthesized by the ecdysial glands or Y-organs. The Y-organ secretes ecdysone, which is converted to 20-hydroxyecdysone, the active ecdysteroid in most crustaceans. Several studies have shown that the Y-organ in some brachyuran crabs also secretes 3-dehyroxyecydsone and 25-deoxyecdysone (summarized in Subramoniam 2000). 25-deoxyecdysone is the precursor to ponasterone A (PoA), the primary circulating ecdysteroid in the premolt stage of the crabs (Subramoniam 2000). Other sources for ecdysteroids are the ovary, epidermis, and the oenocytes (Delbecque et al. 1990). In many crustaceans, molting, and hence somatic growth, continues after maturity, with the result that the Y-organ is active in adults. For most crustaceans, growth and reproduction can be grouped into three functional categories. In the first category, represented by crab and lobster, reproduction occurs after a long intermolt period. The second category includes isopods and amphipods, the growth and reproduction of which are concurrent. The last category relates to the rapidly molting cirripedes, for which reproduction requires several molt cycles. Molting and limb regeneration are intertwined (Fingerman et al. 1998). When limb regeneration occurs, first a limb bud develops within a layer of cuticle, and then becomes free and unfolds when ecdysis occurs as part of the molting process. Synthesis and secretion of ecdysteroid by the Y-organs is inhibited by molt-inhibiting hormone, a peptide that is released from the sinus gland.

31. It has been documented that there is synchronization between reproduction and molting. At least in mysids, accumulation of ovary ecdysteroid takes place during the premolt stage, when the hemolymph ecdysteroid levels rise sharply. It is presumed that the hemolymph ecdysteroids are transported to the ovary along with the yolk precursor material. This trend is seen in other species as well, and shows that the Y-organ is active during premolt and that it produces ecdysteroids that are transported to the ovaries. This observation was confirmed by Subramoniam (2000). A Y-organ ablation was performed on the shrimp, *Lysmata seticaudata*, which caused a subsequent depression of vitellogenin synthesis and retardation in ovarian growth. Further findings on the same shrimp revealed a failure of folliculogenesis, which is a necessary prerequisite for vitellogenin-uptake by oocytes during secondary vitellogenesis.

32. Terpenoids, which are unique to arthropods (Quackenbush 1986), include methyl farnesoate (MF). MF is secreted by a mandibular organ and there is evidence that this compound is involved with the control of ecdysteroid synthesis. When a mandibular organ was experimentally implanted into the shrimp, *Penaeus setiferus*, there was a subsequent shortening of the molt cycle (Subramoniam 2000). Secretion by the Y-organ is controlled by methyl farnesoate, whereas inhibition is exercised by MIH from the X-organ sinus gland. The mandibular organ has also been implicated in the control of reproduction in crustaceans. Mandibular organ implants stimulated ovarian growth in the juvenile spider crab females and methyl farnesoate levels increased in the hemolymph and the mandibular organ during vitellogenesis in the crab, suggesting that this compound has a gonadotropic role similar to that of JH in insects. However, other

studies showed no methyl farnesoate- level effects within the vitellogenic period in the lobster, for example (Subramoniam 2000).

33. Serotonin (5-hydroxytrptamine, 5-HT), a biogenic amine, is one of the most important biologically active substances in animal kingdom as it regulates many physiological and behavioral functions (Moreau et al. 2002). 5-HT has been found in many invertebrate groups, including crustaceans. Moreau et al. (2002) documented its presence in mysids, although they did not study its specific function. Studies reviewed by Quackenbush (1986) suggested that 5-HT induces the release of molt-inhibiting hormone and crustacean hyperglycemic hormone from the eyestalks of decapods.

34. Rather than providing a detailed review of crustacean endocrinology, the few paragraphs presented here summarize the four main types of compounds involved in the regulation of crustacean biology and show the interplay among them in regulating major physiological processes. In summarizing about 75 years of crustacean endocrinological studies, Fingerman (1997) concluded that despite the many significant advances, work in the filed "has really just begun." This is especially true considering the tasks ahead in examining the potential for the disruption of crustacean endocrine systems by anthropogenic compounds.

3.1.7 Echinoderms

35. Echinoderms (e.g., sea stars, feather stars, sea cucumbers) are deuterostomes and are relatively closely related to vertebrates. Therefore, their endocrine systems may have some similarities to those of vertebrates and may share similar targets susceptibilities to chemicals known to have endocrine-disruptive effects on vertebrates (LeBlanc et al. 1999). Vertebrate sex steroids may play a role in echinoderm reproduction (Oberdörster and Cheek 2001).

36. No ecdysteroids or juvenoids are known in echinoderms (echinoderms don't molt). Processes under hormonal, neurohormonal, or local growth factor control include gametogenesis, spawning, growth, and regeneration (reviewed in LeBlanc et al. 1999). Reproduction influenced by steroids and neuropeptides (summarized in LeBlanc et al.).

37. Sea stars synthesize two steroids, progesterone and testosterone, and steroidogenic pathway enzymes (3β-hydroxysteroid hydrogenase, cytochrome P450) occur in sea stars. Estrogen and estradiol synthesis has not been demonstrated, but a receptor for estradiol has been identified. High levels of progesterone in males have been demonstrated at the beginning of spermatogenesis. Therefore, it is very likely that steroids function in sea star reproduction. There is very little information about endocrine functions in other echinoderm groups.

38. Gonad-stimulating substance (GSS), which has been found in radial nerve extracts and stimulates spawning, may be a neuropeptide. GSS indirectly stimulates release of a maturation-promoting factor that readies oocytes for fertilization. Other neuropeptides have been discovered in echinoderms and may regulate feeding.

39. Regeneration is a form of asexual reproduction in some taxa, but also serves to replace lost body parts. Loss of body parts, which may be a defense from predation, most frequently involves arms (e.g., sea stars), but also often may involve sections of epidermis (e.g., sea cucumbers; Kropp 1982). Arm regeneration in feather stars (Crinoidea) occurs by the proliferation of migratory undifferentiated cells and is under nervous system control, which provides the primary regulatory factors (Candia Carnevali et al. 2001b).

3.1.8 Summary

40. The endocrine system of an invertebrate differs from that of a vertebrate organism in the type of endocrine glands present and in the chemical structure (and consequently in the function) of specific hormones that are produced. Invertebrates produce some hormones that vertebrates do not. For example, crustaceans and most other invertebrates produce di- and tri-iodothyronine, but have no thyroid gland, and the function of the thyronines is unknown. Invertebrates use hormones that are not found in vertebrates. Crustecdysone is in some ways analogous to a vertebrate's estrogen hormone, but it is structurally, functionally, and metabolically different from the vertebrate hormone (J.M. Neff, personal communication, January 15, 2002).

3.2 OVERVIEW OF POTENTIAL ENDOCRINE DISRUPTION IN AQUATIC INVERTEBRATES

41. There are two basic types of compounds with the potential to disrupt endocrine systems: synthetic chemicals (xenobiotics) and natural plant chemicals (phytoestrogens) (Crisp et al. 1998). Some examples of xenobiotics are compounds used in plastics (nonylphenol, bisphenol-A), PCBs, and some pesticides. Phytoestrogens include hormone-mimicking substances contained in some agricultural plants and in paper mill effluent. These can be estrogenic or anti-estrogenic.

42. Lafont (2000) described and gives examples of four levels at which EDCs can disturb endocrine systems. At the first level, EDCs block the availability of the precursors required for the synthesis of hormones. The interruption of hormone biosynthesis occurs at the second level. For example, some chemicals inhibit cytochrome P450s, which catalyzed biosynthesis, thus breaking the synthetic pathway leading to hormone production (Lafont 2000). The third level at which an EDC can act is on hormone catabolic processes. In this case, an EDC could act to increase the rate at which a hormone is catabolized, resulting in lower levels in an animal. At the fourth level, an EDC directly interferes with the actions of hormones. EDCs can act as agonists by binding to a hormone receptor and acting as that hormone would to regulate gene transcription (LeBlanc et al. 1999). EDC also may act antagonistically by binding to a hormone receptor without inducing its activity (LeBlanc et al. 1999), thereby obstructing normal endocrine function.

43. The following sections present a brief overview of endocrine disruption in invertebrates that builds on recent reviews by LeBlanc et al. (1999), Oehlmann and Schulte-Oehlmann (2003), and Segner et al. (2003) among others. The overview is not meant to be exhaustive and is organized by major taxon within habitat type. Some information about endocrine disruption in aquatic arthropods, especially crustaceans, is included here, but a more detailed discussion, structured in the context of hormonal effects, is presented in Sections 7, 8 and 9.

3.2.1. Endocrine Disruption in Freshwater Species

44. Porifera: Hill et al. (2002) tested the effects of three chemicals known to disrupt endocrine systems in some animals (ethylbenzene, nonylphenol, bisphenol-A) on two species of freshwater sponges (*Heteromyenia* sp., *Eunapius fragilis*). The study was initiated with gemmules and tested the effects of these chemicals on growth (morphology and rate). Hill et al. found that higher doses caused a morphological abnormality that was similar across chemicals for both species. Germination rates for gemmules in the control treatments were relatively low (70% and <50% for each species) and germination could be inhibited at highest concentrations. Hill et al. could not determine if the observed effects were attributable to a disruption of an endocrine pathway or a more general toxic effect, yet argued that the former was likely (despite the general lack of information about sponge endocrinology).

45. Hydrozoa: Pascoe et al. (2002) examined the effects of 17α-ethinylestradiol and bisphenol-A on the hydrozoan *Hydra vulgaris* and found no physical or physiological damage to the polyps at environmentally relevant concentrations (ng/L) of either chemical. However, they determined that toxicity occurred at relatively high chemical concentrations and concluded that signaling processes necessary for normal development, regeneration, and sexual reproduction were not affected by these estrogenic pollutants at low, environmentally relevant concentrations.

46. Cladocera: Baldwin et al. (2001) documented the effects of several EDCs on the *Daphnia magna*. One experiment focused on exposure to 20-hydroxyecdysone (20-E), the crustacean molting hormone, and to ponasterone-A (PoA), an endogenous compound that has 20 times higher affinity for the ecdysone receptor. The 21-day exposure had little effect on reproduction for either compound, except at the highest concentrations. However, adults suffered high mortality rates and either did not produce broods or produced smaller broods. Second-generation effects were not observed as a result of 20-E exposure, but there was a slightly significant effect on reproduction from PoA exposure. The effect on reproduction could be attributed to the structure of PoA: it has fewer hydroxyl groups, and could be less easily metabolized as is 20-E. The resulting longer exposure could allow second-generation effects. It is also possible that the higher affinity for PoA to ecdysone receptor sites caused a limited effect on secondary vitellogenesis in developing daphnids, which manifested itself as reduced reproduction because of smaller brood size (Baldwin et al. 2001).

47. Recently, many studies conducted by LeBlanc and coworkers have elucidated many aspects of daphnid endocrinology and have subsequently documented the impacts of EDCs. For example, Olmstead and LeBlanc (2002) determined that methyl farnesoate was the likely endocrine factor regulating the development of males in *D. magna*. They later predicted that a juvenile hormone analog, pyriproxyfen, would stimulate the production of males and found that this indeed occurred (Olmstead and LeBlanc (2003). Tatarazako et al. (2003) also found that methyl farnesoate and pyriproxyfen stimulated male production in *D. magna*, even under uncrowded, high-food (i.e., low stress) conditions. They also found that three additional juvenoids—fenoxycarb, methoprene, and JHIII—stimulated male production. The five juvenoids showed similar dose-dependant responses (reduction in fecundity, male production), although fenoxycarb and pyriproxyfen caused the effects at substantially lower concentrations than the other three compounds. Companion studies by Mu and LeBlanc (2002a, 2002b) showed that ecdysteroids are important in regulating daphnid development and that ecdysteroid antagonists, testosterone and fenarimol, interfered with normal development. Testosterone affects early and late developmental stages (acting as a hormone receptor antagonist), whereas fenarimol affected late development (acting as a hormone synthesis inhibitor). Mu and LeBlanc (2004), in a study with more significance to EDC evaluations, examined the potential synergistic effects of a fenarimol-testosterone mixture on daphnid development. Mu and LeBlanc focused on the different actions of each compound, predicting that fenarimol would lower ecdysone levels in daphnids, which would increase the testosterone binding to the ecdysone receptor with the net overall effect of increasing toxicity beyond that of either individual chemical. Their study confirmed this prediction and underscored the importance of considering the potential synergistic of mixtures of chemicals in the environment.

48. Kashian and Dodson (2004) studied the effects of several vertebrate hormones on sex determination and development in *D. magna*. Among the hormones they tested, only long-term (26 days) exposure to progesterone affected sex determination. The second clutch of young produced by *D. magna* exposed to 100 μg/L progesterone contained significantly more males than controls. The effect disappeared with succeeding clutches. Daphnids exposed to 100 μg/L testosterone had significantly reduced fecundity compared to control animals. Two hormones affected daphnid growth. Exposure to diethylstilbestrol (100 μg/L) significantly reduced growth, whereas exposure to gonadotropin (30 μg/L) significantly increased growth compared to controls. Resting egg production and molting were not affected by any of the vertebrate hormones tested.

49. Other studies of endocrine disruption in daphnids showed that styrene dimers and trimers reduced fertility in *Ceriodaphnia dubia* (Tatarazako et al. 2002) and the 17α-ethinylestradiol decreased the number of offspring in *Daphnia magna* (Goto and Hiromi 2003). Goto and Hiromi also found that another contraceptive ingredient, norethindrone, did not affect offspring production or sex ratio in *D. magna*.

50. Amphipoda: Vandenbergh et al. (2003) used a multigenerational assay to examine the effects of sublethal doses of 17α-ethinylestradiol, a synthetic estrogen, on sexual development in *Hyalella azteca*. They compared the second gnathopod size and reproductive tract histology among treated and untreated males and found that F1 males exposed to the lowest 17α-ethinylestradiol doses had significantly smaller second gnathopods than controls, whereas the second gnathopods of those exposed to higher doses did not. This suggested a U-shaped response where effects were observed at a low concentration, but were masked at high concentrations. Vandenbergh et al. also observed cellular abnormalities in males exposed to all concentrations. Among these were larger and more spherical germ cells, a hollow cell structure, and less dense cytoplasm, features that are analogous to female gonad morphology. Oocyte-like structures were detected at some concentrations and smaller spermatids, fewer vas deferens, and irregular spermatogonia were found at highest concentration. The observed histological abnormalities indicated some degree of hermaphroditism in the exposed amphipods. The authors concluded that sublethal exposures of 17α-ethinylestradiol may affect sexual development in *H. azteca*, but they also noted that test concentrations were higher than those reported to affect vertebrates and higher than those observed in natural environments.

51. Watts et al. (2001b) looked at the toxicity of 17α-ethinylestradiol and bisphenol-A and effects of the chemicals on the precopulatory guarding behavior of *Gammarus pulex*. They found that 17α-ethinylestradiol was more toxic than bisphenol-A, but that there was no disruption of precopula or the propensity to reestablish it except at the highest concentrations, which were close to doses that were determined to be acutely toxic. Therefore, these two xenoestrogens had no effect on any endocrine systems that facilitate the behavior. In a follow-on study, Watts et al. (2002) designed a longer study specifically to determine if the lack of response to 17α-ethinylestradiol in the acute test predicted a similar lack of response at the population level in *G. pulex*. The results showed that all of the 17α-ethinylestradiol concentrations that were tested showed population increases greater than those in control treatments (the control treatments did increase over the starting population values). The difference was attributed to the recruitment of neonates and juveniles. The dosed treatments also had more females than the control treatments and had a M:F ratio of ~0.4–0.5:1 versus the ratio of 1:1 attained in the control treatments. The authors concluded that exposure to 17α-ethinylestradiol resulted in significant increase in population size, despite prediction of no effect on reproductive behavior that was derived from the acute assay. The increase in population size was attributed to an accelerated female maturation rate, such that more young were produced earlier, and an increase in number of females. These two studies underscore the need for a chronic assay to develop a more complete understanding of potential EDC effects on populations.

52. Insecta (*Chironomus*): Most of the studies of endocrine disruption in midge larvae has been done on *Chironomus riparius*. In the sole study found during this review that directly concerned endocrine disruption in *C. tentans* (also known as *C.dilutus*), Kahl et al. (1997) conducted an assay to examine the effects on 4-nonylphenol on the life cycle of the midge. The only significant result was on 20-d survival at highest concentration. There were no effects on egg production or viability, emergence time, and sex ratio. Kahl et al. (1997) noted that some egg cases were deformed, particularly at the high nonylphenol concentration.

53. Studies on potential endocrine disruption in *C. riparius* have considered two basic types of endpoints, mouthpart deformities and life cycle reproductive parameters. Deformities of the mouthparts (mentum, mandibles, epipharyngeal pecten) have been observed in association with some contaminated sediments. Physiological disturbance during molting are thought to cause the deformities. Meregalli et al.

(2001) tested the hypothesis that hormonal disruptions during molting contributed to the development of the deformities by subjecting the midge larvae to the known endocrine disrupter 4-nonylphenol. Mentum deformities were significantly more common in larvae exposed to sublethal concentrations of nonylphenol than in the control larvae. The mandibles and epipharyngeal pecten showed few deformities. In a more broadly scoped study, Watts et al. (2003) studied the effects of sublethal doses of the estrogenic endocrine disruptors 17α-ethinylestradiol and bisphenol A on molting and mouthpart structures in *C. riparius*. The highest concentration (1 mg/L) of both chemicals significantly reduced larval weight and delayed molting. Watts et al. noted that the high concentration was environmentally unrealistic and much greater than the concentration known to elicit a response in fish. The remaining doses of either chemical did not affect either parameter. The lowest concentration (10 ng/L) of each chemical caused deformity of the mentum but not the other two primary mouthparts. 17α-ethinylestradiol had a slightly stronger effect than bisphenol A on the mentum. No mouthpart deformities occurred at the highest concentration of each chemical. The mouthpart studies also highlights that classical dose-response curves often do not apply to EDCs, but that inverted U-shaped curves (no/low effects at the concentration extremes, high effects at middle concentrations) more accurately depict the response (Watts et al. 2003).

54. Watts et al. (2001a) used a two-generational study of *C. riparius* to evaluate the effects of 17α-ethinylestradiol and bisphenol-A on the midge life cycle. They found that the main effect of the two chemicals was delay of emergence at higher test concentrations, especially for the second generation. The delay did not affect the typical protandrous emergence pattern in chironomids (males emerge before females). There was no effect on egg production or egg viability. One interesting effect was the alteration of the second generation adult sex ratio where males outnumbered females 2:1 at all but the highest 17α-ethinylestradiol concentration. Watts et al. thought that this effect was aligned with that expected for an estrogenic compound and suggested that this could mean the chemical did not act as an estrogen in this particular case. Bisphenol A did not affect sex ratio.

55. Hahn et al. (2001, 2002) tested the effects of tebufenozide, a pesticide that acts as a molting hormone agonist, on *C. riparius* development and vitellogenin/vitellin (Vg/Vn) levels. The molting hormone agonist was anticipated to accelerate development. Tebufenozide affected development, but that effect did not occur until the final molt from pupa to adult. Hahn et al. pointed out that this effect was counter to that desired for the pesticide's intended butterfly targets, stimulation of an early molt that leads to death. The effects levels determined during this chronic study (e.g., LC50 = 21.14 μg/L) were much lower than those determined for acute exposures. The 2002 study also examined the effects of bisphenol-A and 4-nonylphenol on Vg/Vn immunoreactivity. Males exposed to most contaminant concentrations showed reduced Vg/Vn immunoreactivity. Females were not affected by the contaminants, except the highest bisphenol-A concentration reduced Vg/Vn levels. These results were not expected because bisphenol A and nonylphenol are known to stimulate vitellogenesis in vertebrates. The observed responses were not dose-dependent as all concentrations reduced Vg/Vn somewhat equally.

56. Hahn and Schulz (2002) found that relatively short-term exposure to tributyltin reduced ecdysteroid synthesis in female *C. riparius* larvae at test concentrations as low as 50 ng/L, whereas males showed increased biosynthesis at a concentration of 500 ng/L. Imaginal discs developed more slowly in females, but faster in males at all test concentrations than in the respective control treatments. The authors thought that these gender-specific differences showed that either the ecdysteroid pathways were impacted differently, or that different reactions occurred to the same impact.

3.2.2. Endocrine Disruption in Estuarine and Marine Species

57. <u>Cnidaria</u>: Tarrant et al. (2004) evaluated the effects of exogenous estrogens (17β-estradiol, estrone) on scleractinian coral reproduction. They determined that 29% fewer egg bundles were released by *Montipora capitata* colonies in estradiol-treated colonies than in control colonies. Thus, estrogens seem

to be natural bioregulators in corals, which suggests that exogenous estrogens can reduce coral fecundity. However, the number of eggs per bundle did not differ in treated corals than from corals in control treatments. Growth in *Porites compressa* was significantly reduced in treated corals at estrone levels only slightly greater than ambient levels. However, the mode of action of estrogen in corals is unknown. Tarrant et al. suggested that these results (and others for invertebrates) imply that the potential disruptive effects of estrogens are not limited to interruption of mammalian reproduction.

58. Annelida: The review prepared by LeBlanc et al. (1999) mentioned that there was only one known case of endocrine disruption in annelid worms; juvenile hormone and JH analogs stimulate larval settling and metamorphosis in *Capitella*. In a 78-d study, Hansen et al. (1999) found that the lowest treatment of sediment-bound 4-n-nonylphenol stimulated asymptotic body volume growth and increased mean brood size, but that these effects did not translate to changes in population growth rates. The highest concentration of nonylphenol significantly reduced several reproductive metrics, including brood size, volume-specific fecundity, time to first reproduction, and the growth rate of individuals. The authors did not link the observed effects to possible endocrine disruption, although they noted that the stimulation at the low dose was another case of hormesis, which has been documented for other organisms.

59. Mollusca: The most frequently cited examples of endocrine disruption in mollusks concern imposex, which is an irreversible condition in gastropod snails in which females develop secondary male sex organs (Matthiessen and Gibbs 1998). Imposex, which is most frequently a response to exposure to tributyltin contained in anti-fouling paints, may result from the inhibition of aromatase or other steroid precursors, or from direct impacts to neurohormones (Rotchell and Ostrander 2003). Since the topic has been the subject of very many studies and has been considerably reviewed, it will not be discussed in detail here. Reviews and other significant discussions of imposex are included in Matthiessen and Gibbs (1998), Evans and Nicholson (2000), the series of studies by Oehlmann and others (Oehlmann et al. 2000, Schulte-Oehlmann et al. 2000, Tillman et al. 2001), and Axiak et al. (2003).

60. Nice et al. (2003) studied the effects of single short-term (48 h) nonylphenol exposure of 7-d old larvae of oyster *Crassostrea gigas* on long-term physiological processes. Exposure to environmentally relevant levels of nonylphenol resulted in a sex ratio biased towards females and an increased incidence of hermaphroditism. The exposure also affected gamete viability, severely impacting embryonic and larval development in second generation.

61. Jobling et al. (2003) tested effects of 17α-Ethinylestradiol, 4 *tert*-octylphenol, bisphenol-A, and sewage effluent on egg and embryo production in a common freshwater European snail *Potamopyrgus antipodarum*. All estrogen and xenoestrogen treatments stimulated embryo production, except at the highest concentration, which had an inhibitory effect. These reproductive effects were generally similar to those the authors observed for three fish species (fathead minnow, rainbow trout, carp) in a companion study. Jobling et al. suggested that this snail is a sensitive species whose testing against estrogens may be relevant to estrogenic activity in vertebrates.

62. Crustacea: Marine crustaceans are important organisms to include in the evaluation of the adverse consequences of EDCs, and the selection of suitable species is the one focus of the present review. Early studies of the effects of EDCs on estuarine crustaceans, most of which have occurred within the last few years, have focused on three primary groups, barnacles, copepods, and decapods (Ingersoll et al. 1999, Hutchinson 2002). The results of these studies showed that some crustacean groups were affected by exposure to the tesed EDCs, but others were not and, therefore, extrapolation of the results from testing one crustacean group to another is problematic.

63. *Barnacles*.—Billinghurst et al. (1998, 2000) examined the effects of two estrogens, 4-*n*-nonylphenol and 17β-estradiol on larval settlement and the production of a larval storage protein (cypris

major protein, CMP) in *Balanus amphitrite*. Cyprids use CMP during settlement and the early post-settlement development. Because CMP is structurally related to vitellin, which is analogous to vitellogenin, it can be used as a biomarker of estrogen exposure in lower vertebrates. The expectation in these studies was that cyprid settlement might be affected by the stimulation of CMP synthesis after larval exposure to environmental estrogens. The results of the 1998 study, however, showed reduced settlement after exposure to both estrogens, but that the cause was not related to endocrine disruption. The second study (Billinghurst et al. 2000) measured levels of CMP and found that they were elevated after exposure of nauplii to low levels of the estrogens.

64. *Copepods*.—Hutchinson et al. (1999a, 1999b) found that exposure to several steroids had no effect on the survival and development of harpacticoid copepod (*Tisbe battagliai*) nauplii and cautioned against extending the reported effects of steroid exposure in some species of crustaceans to the group as a whole. At about the same time, Bechmann (1999) showed that high levels (>62 μg/L) of nonylphenol were acutely toxic to *T. battagliai*, but that exposure to a low level (31 μg/L) did not affect any of the measured life-table parameters (survival, sex ratio, fecundity). Breitholtz and Bengtsson (2001) did not find evidence of endocrine disruption in the harpacticoid copepod *Nitocra spinipes* after exposure to the estrogens 17β-estradiol, 17α-ethinylestradiol, and diethylstilbestrol. Chandler and coworkers studied potential endocrine disruption in a third harpacticoid species, *Amphiascus tenuiremis*. Bejarano and Chandler (2003) used chronic multi-generation exposures to evaluate the effects of the herbicide atrazine on reproduction and development in *A. tenuiremis*. While atrazine did not have significant effects on several parameters (e.g., time to maturity, time to egg extrusion, time to hatching), some concentration-related effects occurred. Reproductive failure (mating pairs unable to produce living offspring or females unable to extrude more than one brood) increased with atrazine dose. Nauplii production by F_0 females was reduced at the highest concentration and that by F_1 females was reduced at all concentrations. Both factors combined to reduce total population growth of the F_1 generation at doses lower than those considered “safe” for chronic exposures (26 μg/L). Chandler et al. (2004a) found that sublethal, environmentally relevant concentrations of the insecticide fipronil (and also its degradation products; Chandler et al. 2004b) delayed the maturation of *A. tenuiremis* copepodites to adults, and reduced or virtually eliminated the production of young. These effects were modeled and predicted a 62% decline in the population size of the copepod at the lowest parent concentration tested (0.16 μg/L). Fipronil is a GABA-inhibitor and it is likely that the effects were mediated via disruption of the neural system. Still, a follow-up to Chandler 2004a showed that it was actually only males that were affected at these low levels of fipronil, as indicated by lowered ecdysteroid levels, elevated male vitellogenin, and malfunction in male reproductive ability (Cary et al. 2004). This may consequently be regarded as an example of both neural and possibly endocrine disruption.

65. Copepods probably have been tested against more potential EDCs than any other marine group. For example, Anderson et al. (2001) tested the effects of 14 compounds, including natural vertebrate hormones (17β-estradiol, estrone, testosterone, progesterone), natural invertebrate hormones (20-hydroxyecdysone, juvenile hormone-III), hormone antagonists (flutamide, tamoxifen, hydroxyflutamide), xenoestrogens (17α-ethinylestradiol, 4-octylphenol, bisphenol-A), and environmentally relevant compounds (nonylphenol ethoxylate, diethyl phthalate) on larval metamorphosis (nauplius to copepodite) in the calanoid copepod *Acartia tonsa*. The important points of this study were that chemicals differed in their relative effects on survival and larval development. Some of the chemicals tested affected development at concentrations well below those determined to be toxic. Others delayed development only at concentrations close to those that were toxic, thus the main effect of the chemical was likely toxicity. Chemicals having very similar effects on toxicity, and having similar octanol/water partition coefficients (K_{ow}), could have very different effects on larval development. For example, flutamide and testosterone have the same K_{ow} and had similar effects on copepod survival, but flutamide was a much stronger inhibitor of larval development than testosterone.

66. *Amphipods*.—There have been few studies of endocrine disruption in marine amphipods. Brown et al. (1999) found that exposure to 4-nonylphenol reduced growth in the gammaridean amphipod *Corophium volutator*, but that this was probably a general response to the exposure rather than an interaction with molting hormones. They also reported that males exposed to 4-nonylphenol had larger second antennae than those in control treatments and suggested that the compound may have acted on the androgenic gland. Field studies suggested that intersexuality observed in an estuarine amphipod, *Echinogammarus marinus*, might be indicative of endocrine disruption, although a causative link was not established (Ford et al. 2004a, 2004b).

67. *Mysids*.—Mysid crustaceans have been used in regulatory (and other) toxicity testing for more than 20 years. Standard testing protocols have been developed for some species. Despite that little is known about general endocrine functions in mysids (Ingersoll et al. 1999) and there have been few direct links between potential EDCs, beyond certain IGRs, and endocrine disruption in mysids, they have been suggested as providing a useful model of the hormonal control of crustacean molting (Cuzin-Roudy and Saleuddin 1989). McKenney and Celestial (1996) studied the effects of methoprene, which is a JH analog used to control mosquitoes, on *Americamysis bahia* and found that mysids grown at sublethal concentrations were smaller, had a longer time to the production of the first brood, and produced fewer young per female than control animals. They suggested that the effects shown were likely from the interruption of endocrine function by the methoprene.

68. Advances in biological control agents to control insect pests have inspired the synthesis of insect growth regulators (IGR), which find their way into the estuarine environment by either direct or indirect application. Crustaceans, which along with insects are in the phylum Arthropoda, could also be sensitive to these compounds. The mysid group has been shown to be among the most sensitive members of the estuarine community (McKenney 1982, 1985, 1986, 1996; Nimmo and Hamaker 1982; Nimmo et al. 1981). One study focused on exposure of *Americamysis bahia* to methoprene, a JH analog (McKenney and Celestial 1996). The goal was to determine whether typical application rates shown to control mosquito larvae also cause problems for non-target organisms. The results showed a significant effect during the mysid life cycle test. Total lethality occurred at 125 μg/L in a 4-day test. Similar concentrations caused significant mortality in the larvae of an estuarine crab and shrimp. Other sublethal endpoints, such as reduced growth (weight), longer time to first brood, and a significant reduction in brood size were also observed. These results suggest that methoprene could interfere with the endogenous endocrine system, which uses hormones that act like JH. Retarded growth rates were also accompanied by bioenergetic disruption, resulting in lower net growth efficiency values. This suggested that increased metabolic demands reduced the amount of assimilated energy available for new tissue production (McKenney 1982, 1985). The delays in mysid first brood production could be the result of slowing sexual maturity and/or embryogenesis. Diminished reproductive success could be the result of inhibited vitellogenesis, modifications in ovarian development, or disruption of successful embryogenesis. In either case, further work is required with the mysid to determine a more conclusive cause-and-effect relationship between potential EDCs and their effects, as observed by test measurement endpoints.

69. Verslycke et al. (2002, 2003a, 2003b, 2004b) studied testosterone metabolism by mysids (*Neomysis integer*) and examined the changes in energy and testosterone metabolism after exposure to potential EDCs. Because these studies focused on potential endpoints, they are presented in more detail in Section 6.4.3.

70. *Decapods*.—Several studies have investigated the effects of EDCs on crustacean life cycles using decapod larvae as the test organisms (e.g., Lee and Oshima 1998; McKenney et al. 1998; Nates and McKenney 2000). Several studies (reviewed by McKenney 1999) have reported effects of the exposure of decapod larvae to JH analogs suggestive of the interruption of endocrine processes, but direct links were not established. More recently, Nates and McKenney (2000) found that exposure to the pesticide

fenoxycarb disrupted lipid metabolism in mud crab larvae and suggested that the compound could be interfering with the endocrine regulation of lipid metabolism. Exposure to fenoxycarb delayed maturation of xanthid crab (*Rhithropanopeus harrisii*) larvae by about 25% compared to controls (Cripe et al. 2003). Some evidence of endocrine disruption in decapods was provided by Snyder and Mulder (2001) who found that exposure of lobster (*Homarus americanus*) larvae to the pesticide heptachlor altered ecdysteroid hormone levels that were linked to delays in molting. Metals such as mercury, cadmium, and zinc have been reported to affect molting and limb regeneration in crabs. Organic compounds such as Aroclor 1242 and sodium pentachlorophenate reportedly had a similar effect–inhibition of limb regeneration–in the grass shrimp, *Palaemonetes pugio*, but had no effect on the molting cycle. This suggests that these chemicals act directly on limb development, but not on the hormonally controlled molting cycle (Fingerman et al. 1998).

71. Echinodermata: Candia Carnevali et al. (2001a, 2001b) studied the feather star *Antedon mediterranea* (Crinoidea) arm regeneration in response to PCB exposure as endocrine disruptor effect. They found that the early phases of regeneration were not different in feather stars exposed to PCBs than in those from control treatments, but noticeable effects occurred later (first at ~7 d, more extensive effects at ~14 d). The primary effect observed in exposed treatments was increased growth rate. Effects, including rearrangement and/or dedifferentiation of some tissues in the regenerating stump, also occurred at cellular level. No abnormal histological effects on feather star arms were observed. The authors concluded that the observed growth and tissue/cellular effects from the exposure PCBs were consistent with pseudoendocrine activities and steroid dysfunction.

3.2.3 Summary

72. Rather than summarize the various impacts, or lack of impacts, of the EDCs that have been studied to date, this section will highlight some of the general findings that are important to consider.

- Chronic assays often reveal impacts by a chemical at doses that are much lower than those eliciting effects during acute exposures. This emphasizes the importance of chronic assays in an EDC evaluation program (e.g., Watts et al. 2001b, 2002).
- Potential EDCs may have effects in invertebrates other than those anticipated by knowledge of their actions in vertebrates (e.g., Hahn et al. 2002, Watts et al. 2001b).
- Pesticides that target certain aspects of insect physiological process may affect non-target organisms in ways not predicted by the pesticide's desired action (e.g., Hahn et al. 2001, 2002).
- Males and females may react differently to chemicals that are not thought to cause gender-specific responses (e.g., Hahn and Schulz 2002).
- Chemicals that have very similar effects on toxicity, and have similar octanol/water partition coefficients (K_{ow}), can have very different effects on endocrine-controlled processes (e.g., Anderson et al. 2001).
- Some of the endpoint responses (e.g., larval development, ecdysone balance) measured in a study of one or more potential EDCs may also be caused by non-EDC stressors (e.g., food quality/quantity, hypoxia, thermal stress).

73. Finally, and perhaps most importantly, chemical mixtures may show synergistic impacts that are not evident in the actions of the individual components (e.g., Mu and LeBlanc, 2004). In nature, most animals will almost certainly be exposed to chemical mixtures rather than to single compounds, thus considering the potential effects of mixtures is important to an EDC evaluation program. However, to be

able to predict the possible outcomes of mixtures, at a minimum the individual impacts and mechanisms of action of the component chemicals need to be understood.

3.3 EXTRAPOLATION ISSUES

74. The traditional practice in toxicology of testing the effects of a stressor on individuals from one or a few species has raised two major issues that are of concern to an EDC evaluation program. Simply put, can data collected from tests involving individuals of a species be used to predict potential impacts to other species, or to populations? These two extrapolation issues are discussed in this section.

3.3.1 Extrapolation from Taxon to Taxon

75. It is not practical or even possible, to conduct toxicity tests on every species in a particular ecosystem (e.g., Hutchinson 2002), and therefore, the reasonableness of extrapolating the effects of a stressor (e.g., an EDC) observed for one species to another is an important concern (Segner et al. 2003). There are several approaches to establishing the transferability of the results from testing one species to another. One method is to compare the structural and functional similarity of endocrine systems and/or hormones among taxa. For example, Oberdörster and Cheek (2001) reviewed studies on the ecdysteroid systems of various arthropods, mentioning that the structure of ecdysone in crustaceans is identical to that in insects and that the primary function of the hormone in both groups is to regulate molting. They concluded that information on this system in one arthropod species should be applicable to another. More support for this type of argument is that there is some evidence that peptides are conserved through various evolutionary lineages from invertebrates to vertebrates (Anctil 2000). This could be interpreted to mean that effects on particular ecdysteroid or peptide systems in one species are likely to be the same as in another species that has the same or very similar system. There are several issues that render this reasoning inadvisable. Lafont (2000) stressed that one of the key general observations concerning invertebrate endocrine systems was that molecules may be structurally related and yet have very different functions in different taxa. Chang et al. (2001) reviewed many studies and showed that the same hormone may have functions that differ among the different life stages of an individual. Finally, even if a hormone can be shown to have the same function in a taxon, the sensitivity of that hormone to disruption by a chemical could vary greatly among taxa. For example, Watts and Pascoe (2000) who clearly demonstrated that *Chironomus tentans* (also known as *C.dilutus*) and *C. riparius* differed significantly in their responses to the same chemical stressor. These observations argue for caution in the use of data from a few species to craft generalizations about many (Lafont 2000).

76. Despite this difficulty in extrapolating from one taxon to another, a program to evaluate potential EDCs can be successful if it selects a sensitive taxon that is ecologically relevant (Nimmo and Hamaker 1982). This approach may not offer protection to all species, but should at least offer some degree of protection to critical parts of the ecosystem that have similar exposure probabilities. The program should also develop robust test protocols (Hutchinson 2002) that can be used efficiently by many laboratories and eventually should include representatives from several taxonomic groups (Hutchinson 2002, Oehlmann and Schulte-Oehlmann 2003).

3.3.2 Extrapolation from Individuals to Populations

77. The toxicological effects of chemicals, including potential EDCs, as measured during tests of exposure to individuals may not have the same potential to predict possible impacts to populations because the population response may not occur at the same exposure levels as the individual response or may be masked by another stress response. Therefore, the challenge posed to programs charged with assessing the potential risks of EDCs is to establish likelihood that a chemical, or suite of chemicals, will have adverse effects on populations (Crisp et al. 1998, Gleason and Nacci 2001). Thus, the critical need is to be able to

detect specific cause-and-effect relationships between a chemical and the responses observed in the field when several factors may cause similar responses (Crisp et al. 1998).

78. Two general approaches to extrapolating from EDC effects on individuals to potential impacts to populations have been identified. One approach uses data gathered during bioassays to attempt direct extrapolation to populations. The other approach uses population modeling techniques to connect effects observed on individuals to potential population impacts.

79. As an example of the first approach, Watts et al. (2002) used survival and sublethal endpoints (growth, development, reproduction) to attempt to predict effects at higher levels of organization. They used a life table or demographic study where they recorded age-specific mortality and fecundity of individuals during life span of a single cohort and used those data to estimate the intrinsic rate of population increase. Watts et al. (2001b) had done an earlier acute study that showed no effect of 17α-ethinylestradiol on the freshwater amphipod *Gammarus pulex*. A subsequent chronic exposure resulted in changes in maturation rate and sex ratio that translated to measurable effects at the population level (at least in the laboratory) (Watts et al. 2002). Watts et al. concluded that chronic tests provide a more integrated approach to predicting population responses.

80. McTavish et al. (1998) presented a general modeling approach to the evaluation of potential EDCs. McTavish et al. mentioned that many EDCs may persist in natural environments for some time because they are lipophilic and have fairly slow decay rates. These persistent EDCs may change mortality, reproductive, and life-stage transition rates, might become noticeable only after some delay, could affect different life stages present, and may affect offspring of exposed individuals. This introduces a level of complexity into EDC evaluations that may interfere with efforts to use typical toxicity test data to predict population-level effects. Mathematical models are useful tools to investigate these complex dose-response relationships (McTavish et al. 1998, Gleason and Nacci 2001). McTavish et al. built a general model that enabled mortality, reproductive, and life-stage transition rates to be evaluated alone or in combination. The model also allowed delayed responses and transgenerational impacts to be analyzed. The model evaluated the effects of two dosing scenarios; a single pulse of a chemical into a system and its subsequent decay, and continuous dose of a chemical. McTavish et al. argued that the effective dose response should be based on chemical concentrations in the test animals rather than those in the water because traditional dose-response assessments based on water concentrations may not detect all potential risk to natural systems. This approach probably is not very practical for many invertebrates, especially small arthropods because the amount of tissue required for the analyses would significantly increase the number of animals required for the test. The authors concluded that models are very useful for examining the changes from chemical exposure to the effective dose, and from that dose to populations. Models can synthesize laboratory toxicity test data and use them to extrapolate to a composite picture under a variety of dosing regimes and also can be used to assess the relative importance of various bioassay endpoints. Combinations of stressors can act synergistically to cause significant effects on individuals or populations even though the individual compounds did not when tested separately (Arnold et al. 1996, Mu and LeBlanc 2004). These synergistic effects can be evaluated by the model that McTavish et al. used.

81. Kuhn et al. (2000, 2001) applied the general principals proposed by McTavish et al. in a general evaluation of the ecological relevance of mysid toxicity tests using *Americamysis bahia*. Kuhn et al. (2000) used a model to predict the concentration of a contaminant that would result in no population growth and compared that concentration to standard toxicity test data. They also evaluated which test endpoint, survival or reproduction, was better at predicting population responses. Later, Kuhn et al. (2001) evaluated the ability of their age-classified projection matrix model to predict the population response of *A. bahia* over more than three generations maintained in the laboratory. Data incorporated into the model were gathered from daily records of survival and reproduction, which begins at about Day 17, during toxicity testing with several types of chemicals (e.g., metals, organic compounds). The analyses showed

that the population growth rates were dependent on concentration for most of the chemicals tested. Kuhn et al.'s comparison of endpoints showed that the LC_{50} estimated from a 96-h exposure and the chronic life-cycle test endpoints were highly correlated to population changes (r = 0.96 and 0.93, respectively). However, linear regression analysis showed that the life-cycle test was better than the LC_{50} at predicting population-level effects. Within the chronic assay, reproduction was more strongly correlated than survival to population growth rate (r = 0.98 and 0.79, respectively). The model was verified (Kuhn et al. 2001) by using the results of a 28-d life-cycle test conducted with *para*-nonylphenol as the toxicant, to generate data that allowed the model predictions of mysid abundances at the end of the multigenerational (55-d) assay. The predicted abundances then were compared to actual abundances from the assay. The results showed that the model could predict population effects "reasonably well" (Kuhn et al. 2001) in the laboratory. The model-predicted concentration (16 μg/L) at which no population growth would occur did not differ significantly from that derived from the multigenerational test data (19 μg/L). The data from the 28-d test also were used to calculate a chronic exposure value (12 μg/L), which was compared to the 55-d "no-growth" concentration. The lower value was selected as the conservative concentration at which the population should be protected from exposure to *para*-nonylphenol.

82. While either or both approaches may be appropriate, the important consideration is that if population-level impacts are ignored, the probability that the direct effects of EDCs on ecosystems will be underestimated increases (McTavish et al. 1998).

4.0 AQUATIC ARTHROPODS IN THE EVALUATION OF POSSIBLE ENDOCRINE DISRUPTION

83. Many anthropogenic pollutants eventually end up in the world's oceans, carried there through riverine and estuarine pipelines (Nimmo and Hamaker 1982). Since the mid-1990s, there is an increased awareness that many sewage constituents or chemicals associated with industrial production that enter the environment can disrupt endocrine systems, and that these compounds will likely affect marine organisms (Depledge and Billinghurst 1999; Oberdörster and Cheek 2001). Although early concern over EDCs focused on vertebrates, attention recently has broadened to include invertebrates because they are ecologically important and have distinctive endocrine systems that differ from those of vertebrates. Estuaries, which are intrinsically and commercially important ecosystems, are among the earliest recipients of EDCs. Among the many estuarine organisms that could be adversely affected by these compounds, crustaceans are good candidates for study of potential impacts. Some of these species are discussed in this section. Attention here is focused on aquatic arthropods (insects and crustaceans) because they are often among the most abundant organisms in freshwater and estuarine systems, form vital links in estuarine food webs, and are known to be susceptible to the effects of EDCs.

84. Hutchinson et al. (2000) offered a counter argument to the inclusion by the EDSTAC and EDSP of an arthropod, specifically a daphnid or mysid, in the endocrine screening program at this time. The principal objection applicable to both taxa was the general data gap in the basic endocrinology of mysids and daphnids. They argued that the assertion by EDSTAC that vertebrate estrogen or androgen disruptors could interfere with ecdysteroid activity was only speculation and that any effects on reproduction or development by known vertebrate EDCs have not been directly linked to ecdysteroid or juvenile hormone activity. This is indeed an important issue to consider in this context, but at the same time, the understanding of invertebrate endocrine systems is rapidly improving (Section 3.0) and the links between hormones and processes in crustaceans are constantly being established. For example, in companion studies, Mu and LeBlanc (2002a, 2002b) showed that ecdysteroids regulate the embryonic development of daphniids and that this development could be interrupted by testosterone and fenarimol, a fungicide used in agriculture. Testosterone did not display ecdysteroidal activity, but interfered with ecdysteroids by outcompeting them to occupy the ecdysteroid receptor (Mu and LeBlanc 2002a). Fenarimol acted by reducing body levels of ecdysone in individual daphnids (Mu and LeBlanc 2002b). Vandenbergh et al. (2003) found that the synthetic estrogen 17α-ethinylestradiol altered sexual development in individuals of the amphipod *Hyalella azteca*. Males did not develop secondary sex features, reproductive system morphology was altered, and sex ratios in exposed groups favored females. Also, recent studies of crustaceans have identified that methyl farnesoate has a role in regulating larval development in lobsters (summarized by Chang et al. 2001) and stimulates gonad development in a freshwater prawn (Nagaraju et al. 2003). The industrial chemical bisphenol-A has also been shown to to delay development of young stages in calanoids, harpacticoids and cladocerans (Andersen et al. 2001; Marcial et al. 2003; Mu et al. 2005) As a disrupting mode of action, bisphenol-A was supposed to exert its activity via an enhancement of the activity of methyl farnesoate (Mu et al. 2005), thereby decreasing larval and juvenile development. As Verslycke et al. (2003a) stated, the discussion over whether or not an observed effect is directly related to endocrine disruption or not may be appropriate, but neither that discussion, nor the cause of the effect, is of concern to population maintenance of the affected organism.

4.1 INSECTA

85. Although many insect species might be chosen for use in EDC evaluations the focus in this section is on the chironomids, which are ecologically important, have had well-developed testing protocols

prepared and evaluated, and have been widely used in general toxicological testing in addition to endocrine disruptor screenings.

4.1.1 Midge Larvae (Chironomus tentans **[or** ***C. dilutus*****] and** ***C. riparius)***

86. Natural History: *Chironomus* spp. are true midges (Order Diptera) that have a widespread worldwide distribution (Pennak 1989). Adult midges are small, nonbiting, and swarm near bodies of water at night. Larval stages are aquatic, inhabiting fine tubes in the upper layers of the sediment. Midge larvae feed on algae, higher plants, and organic detritus (Pennak 1989). Chironomid larvae are ecologically relevant for toxicity testing because of their widespread distribution, numerical abundance, and importance as prey for juvenile and adult fish. Two species of *Chironomus* are typically used in toxicity testing; *C. tentan*s in North America and *C. riparius* in Europe. *Chironomus tentans* eggs hatch about 2 d to 6 d after laying and begin the first of four aquatic instar larval stages (summarized in Benoit et al. 1997). The larval development lasts about 23 d and is followed by a short (~1–2 d) pupal stage. Males emerge about five days before females (protandry). Adults are short-lived, about 7 d. The aquatic development period for *C. riparius* is shorter as adults emerge within about 15–17 d after beginning the first instar stage (Watts and Pascoe 1996, Watts et al. 2001a) and live about 4 d (e.g., Ristola et al. 2001). This was confirmed in a direct comparison in natural and artificial control sediments between the two species (Watts and Pasco 2000).

87. Availability, Culture, and Handling: Both species of *Chironomus* are readily available from commercial sources and do not need to be collected from the field. They are easily maintained in laboratory culture. Detailed culture requirements for *C. tentans* have been described by EPA (2000). Larval are held at about 23°C in glass aquaria (e.g., ~19 L volume containing ~8 L water) with finely shredded paper toweling or silica sand used as substrate. About 600 eggs per 8 L should be stocked into each aquarium. High densities of larvae increase development time, delaying adult emergence. Larval are fed a slurry prepared of commercial fish food flakes at a concentration of ~0.04 mg dry food/mL culture water. Adult emergence commences in about three weeks after egg hatching (23°C). Chronic tests must be initiated with <24-h old larvae, therefore, laboratories must maintain cultures to ensure larvae of the correct age are available for testing.

88. McCahon and Pascoe (1988) presented information on culturing *C. riparius*. Conditions for culture are somewhat similar to those for *C. tentans*. The substrate is based on homogenized cellulose paper (filter paper). Cultures are maintained at 18°C to 20°C and are fed a ration of commercial fish food flakes daily.

89. Strengths and Weaknesses: The principal strengths of using *Chironomus* in toxicological testing are the high ecological relevance, ready availability, and ease of culturing of the two primary species. Formal test procedures for both species are well-developed and the species have been used in a variety of laboratory (e.g., Benoit et al. 1997, Environment Canada 1997a, US EPA 2000) and *in situ* (Castro et al. 2003) testing situations. At least one other species, *C. prasinus*, has been used in some EDC testing (Sánchez and Tarazona 2002). The life cycle of chironomids is relatively short and amenable to multigenerational testing. The life cycle of *C. tentans* is about 33–38 days (Benoit et al. 1997); that of *C. riparius* is likely shorter (Watts & Pascoe 1996, Watts et al. 2001a). Recent studies have included several evaluations of potential endocrine disrupting chemicals on *C. riparius* (Hahn et al. 2001, Watts et al. 2001a, Hahn et al. 2002, Hahn and Schulz 2002) and *C. tentans* (Kahl et al. 1997). One possible weakness is the apparent lack of response, or conflicting responses, of life cycle endpoints in *C. riparius* to exposure to two known xenoestrogens, bisphenol-A and 17α-ethinylestradiol, or in *C. tentans* exposed to the surfactant 4-nonylphenol (Kahl et al. 1997). Chironomid larvae are sediment dwellers; therefore the testing protocol must provide the test animals with a suitable substrate. Watts and Pascoe (2000) showed the type of sediment (artificial or natural) was an important contributor to the differences in response that

they observed. Concentrations of contaminants were greater in the porewaters of the artificial sediment than in those of the natural sediment.

90. Another important issue that must be considered when using either species of *Chironomus* is that the two were shown to have different sensitivities to the same toxicant. Watts and Pascoe (2000) found that *C. tentans* was more sensitive than *C. riparius* to the same toxicants evaluated under the same test conditions. They also determined the *C. tentans* was less physically robust than *C. riparius*, which led to increased variability in the data for *C. tentans*, especially emergence data (there was poor emergence in control animals). The response criteria were affected by type of sediment, the choice of species, the particular toxicant, and the duration of the experiment.

4.2 CRUSTACEA

91. Although insects, crustaceans, and pycnogonids (sea spiders) are the primary arthropod taxa that occur in estuarine and marine communities, crustaceans are certainly the more abundant and, therefore, the more ecologically important group. Many insect species occur in these waters but they are relatively poorly studied. Little is e.g. known about pycnogonid biology and endocrinology. Thus, the focus in this section is directed towards the freshwater, estuarine and marine crustacean species that so far have been identified as likely to be amenable to studies on development and reproduction as well as specific EDC evaluations.

4.2.1 Amphipods

Hyalella azteca Saussure 1857

92. Natural History: Historically, *Hyalella azteca* has been considered a very common inhabitant of permanent fresh water systems throughout North, Central, and northern South America (Bousfield 1973, Pennak 1989, Gonzalez and Watling 2002). As discussed in the "Strengths and Weaknesses" section below, the species' actual distribution is probably much more restricted. *Hyalella* inhabits a variety of unpolluted springs, streams, brooks, pools, ponds, and lakes (Pennak 1989), but also may occur in large coastal rivers and into tidal fresh waters (Bousfield 1973). These epibenthic-burrowing amphipods may occur in large numbers; they are detritivorous, feeding primarily on algae and bacteria. *Hyalella* produces about 18 eggs per brood although the number can vary with female age and size (Pennak 1989). At warmer temperatures (24°C–28°C), eggs hatch in about 5–10 d and females can produce about 15 broods every 150 days (Pennak 1989, EPA 2000).

93. Availability, Culture, and Handling: *Hyalella azteca* has been used in toxicity testing for many years and is widely available from many commercial suppliers. US EPA (2000) provides a detailed description of culture requirements. The amphipods can be cultured in containers of various sizes (e.g., 2L–80L) at a water temperature of about 23°C and a light:dark photoperiod of 16:8 h. The water should be renewed periodically and monitored for various parameters such as hardness, alkalinity, pH, and ammonia. Amphipods can be fed commercial fish food flakes, or a combination of yeast-Cerophyl®-trout chow (YCT, prepared according to the recipe in US EPA 2000 or purchased commercially) and the alga *Selenastrum capricornutum*.

94. Strengths and Weaknesses: *Hyalella azteca* is an ecologically relevant testing organism. It has been used in many types of toxicity testing and several protocols, including life cycle testing, to guide testing have been written for it (e.g., Environment Canada 1997b, US EPA 2000). This amphipod is readily available and relatively easy to culture. However, endocrine disruption evaluations, and any other toxicity evaluations, with this species are hampered by recent revelations that the widespread "species" *Hyalella azteca* is actually a mixture of many species. Several studies, beginning in the late 1990s, have

investigated various populations of *H. azteca* from around North America. Duan et al. (1997) studied six lab "populations" of *H. azteca*; one from Burlington, Ontario, one from Conesus Lake NY; and four originally derived from the Corvallis, Oregon population [the Nebeker strain that was originally collected in 1982 (Duan et al. 2000b)], and another species in the genus, *H. montezuma*. They found that the four populations derived from Oregon were relatively closely related and distinct from the other two and from *H. montezuma*. Duan et al. concluded that the populations represented four species (including *H. montezuma*). McPeek and Wellborn (1998) found allelic and genotypic differences between large-bodied and small bodied ecotypes of *H. azteca* in southeastern Michigan. They determined that the two ecotypes did not interbreed and that it was likely that they represented different species. Duan et al. 2000a studied 12 populations, all presumably *H. azteca*, from geographically isolated parts of N. America. They used electrophoresis to identify nine groups. The large genetic differences that were identified led them to conclude that all nine probably represent different species. Witt and Hebert (2000) studied populations from wide geographical area in NA, most of which were from around the Great Lakes (24 lakes and ponds in Ontario and Wisconsin), but also included the Yukon and New Brunswick. They found low levels of gene flow, reduction in genetic variability, low heterozygosity, unique alleles, and strong genetic differentiation and divergence among populations, concluding that there were at least seven mitochondrial lineages. Gonzalez and Watling (2002) conducted a morphological study of type specimen of *H. azteca* and compared it to specimens from Maine, Texas, Mississippi, Michigan, Oklahoma, and Hawaii concluding that *H. azteca* is a species complex. Thus, the taxon currently known as "*Hyalella azteca*" is probably comprised of at least a dozen species. Gonzalez and Watling (2002) recommend that any further toxicological studies confirm identification of species. If animals are obtained from a commercial supplier, the original source of that supplier's culture should be identified.

95. The potential ramifications of using different genetic species on toxicity testing was underscored by Duan et al. (2000b, 2000c) who tested the effects of various metals, pH, and fluoranthene on different genotypes within a population of *H. azteca* derived from the Nebeker strain. They discovered that some genotypes were more susceptible to the stressors than others, and that the susceptibilities differed across genotypes. This differential susceptibility implies that a population that has a higher frequency of a particular genotype that happens to be resistant to a particular stressor will show better survival than a population that has a high frequency of a genotype that is susceptible to the stressor. That is, different genetic populations may provide different responses to the same stressor.

Gammarus pulex

96. Natural History: *Gammarus pulex*, an amphipod that is also known as scud, is widespread throughout Europe, where it is often the most abundant invertebrate in streams. *G. pulex* is a primary prey item of fish and used in biotic indices to describe pollution in rivers (summarized by McCahon and Pascoe 1988). The reproductive biology of *G. pulex* involves precopulatory guarding of the female by a male (e.g., Watts et al. 2001b). The male clasps onto a female and swims with her until she molts to assuring sexual contact during the time at which mating can occur. This particular behavior has been developed into a bioassay procedure (Poulton and Pascoe 1990, Pascoe et al. 1994). It is likely that a chemical signal facilitates this behavior, although evidence for pheromonal control of mating in this species is contradictory (summarized by Watts et al. 2001b). A similar assay was developed for the estuarine gammarid, *Gammarus duebeni* (Lawrence and Poulter 1996).

97. Availability, Culture, and Handling: *G. pulex* is available from at least one commercial supplier, but it is not clear if those animals are cultured by the supplier or field collected. Most studies report using field-collected animals and hold them for a short time before testing. McCahon and Pascoe (1988) developed culture methods for *G. pulex*. Gravid females and pre-copula pairs are removed from the collection and placed in breeding chambers (1-L plastic jars with an open, mesh-covered bottom to allow juveniles to escape); these chambers are placed into larger (2–8 L) tanks that are provided with flow-

through dechlorinated water (the chambers can be maintained static, if water is renewed periodically). Adults are fed a variety of conditioned tree leaves (*e.g.,* horse chestnut, elm, sycamore, or oak) that are collected during fall, air dried, and stored until just before required for feeding. The leaves are prepared by being placed in organically enriched dechlorinated water to promote the bacterial and fungal growth that provides an important food for *G. pulex*. About 200 gravid females in a chamber will provide enough juveniles (500–1000) to stock the rearing tank. Juveniles are fed conditioned leaves, which are supplemented with adult feces from the adult containers. Timing can be arranged to provide different age ranges for testing. Culture temperatures were not provided, but at 13ºC about 70% of juveniles reach maturity in 130 days, after completing 10 molts.

98. Strengths and Weaknesses: *Gammarus pulex* provides a test organism that has considerable ecological relevance in European freshwaters. Because organisms are usually field collected, the potential for population-related differences in pollutant sensitivities is high and should be assessed before comparing the results of tests performed on different populations. However, collected animals are relatively easily maintained in culture systems once established and can provide a suitable number of test organisms for some time. *G. pulex* has been used in many types of bioassay procedures, including behavioral (Poulton and Pascoe 1990, Pascoe et al. 1994) and *in situ* (Matthiessen et al. 1995) testing. However, formal chronic test protocols have not been developed (Segner et al. 2003).

Leptocheirus plumulosus:

99. Several species of benthic estuarine and marine amphipods are used regularly in regulatory toxicity testing, including *Ampelisca abdita*, *Eohaustorius estuaries*, *Rhepoxynius abronius*, and *Leptocheirus plumulosus*. Test guidelines have been established for these species (e.g., US EPA/USACE 1998, US EPA 2001). Several other species have been used in studies of the toxic effects of various contaminants. Among these are *Corophium volutator*, *Gammarus* spp., *Microdeutopus gryllotalpa*, *Echinogammarus marinus*, and *Grandidierella japonica*. Among these amphipods, *L. plumulosus* probably is the best suited for adaptation to an EDC testing program and, therefore, is discussed in more detail below.

100. Natural History: *Leptocheirus plumulosus* is a common infaunal amphipod occurring in estuaries along the east coast of the U.S. from northern Florida to Massachusetts (Bousfield 1973). *L. plumulosus* lives in simple burrows it digs into the upper layers of the sediment. It is a detrital feeder, extracting organic material that is suspended in the water and from ingested sediment. *L. plumulosus* tolerates a wide range of salinities, from 1‰ to 35‰ (US EPA 2001). Its generation time is short, requiring about 24 days at 23°C (US EPA 2001). Another species in the genus, *L. pinguis*, is known to use parental care, sheltering juveniles in its burrows (Thiel 1997). *L. plumulosus* has become increasingly selected as the test species in contaminant sediment evaluations. A chronic survival, growth, and reproduction testing protocol for the species was recently developed (US EPA 2001).

101. Availability, Culture, and Handling: *L. plumulosus* is readily available from several commercial suppliers who maintain large cultures of the species. Laboratories can obtain amphipods having a wide variety of ages. Laboratories conducting the chronic testing protocol need to culture the amphipods in-house because the protocol requires that the test be initiated with neonates (<48-h old). Cultures are easy to start and are maintained at temperatures of 20°C to 25°C and at the salinity that will be used in testing, which is usually 20‰ (US EPA 2001). Cultures are fed finely milled fish food flakes and the culture water is renewed three times per week. Cultures may have to be thinned because these amphipods are very prolific. Cultures require about 6 weeks maturing sufficiently to provide enough neonates for testing.

102. Strengths/Weaknesses: The principal strengths of *L. plumulosus* for EDC evaluations are its ready availability, ease of culture, and sensitivity to contaminants. The species is becoming widely used in

toxicity testing programs. A 28-day chronic testing protocol, which could be adapted to EDC testing programs, has been developed for the species (Emery et al. 1997, US EPA 2001). Some aspects of the species' reproductive endocrinology have been studied (Volz et al. 2002, Block et al. 2003) and the impacts of some chemicals known to affect endocrine systems have been tested on the species (Lussier et al. 2000, Zulkosky et al. 2002). Spencer and McGee (2001) used data collected from field populations of *L. plumulosus* to develop a stage-structured population model that allows extrapolation from laboratory toxicity tests to population impacts. McGee and Spencer (2001) tested the model by using laboratory testing data to project the effects of sediment toxicity on the population growth rate. They found that the population projections were very similar to amphipod abundances observed at the site from which the sediment was collected. *L. plumulosus* abundance in the area from which the sediment was collected. *L. plumulosus* occurs only along the east coast of the U.S. and, therefore, serves only as a surrogate species for estimating the impacts of EDCs on faunas from other regions.

4.2.2 Daphnids

103. Natural History: These three species of daphnids are widespread, occurring in freshwater ponds or lakes throughout much of the world (Weber 1993). *C. dubia* is a small species commonly found among plants in the littoral zone of lakes and ponds. *D. magna* lives primarily in lakes where the hardness exceeds 150 mg/L $CaCO_3$, whereas *D. pulex* lives in clean, well-oxygenated ponds (Weber 1993). Much of the year, when environmental conditions are favorable, populations of these species consist primarily of females; males are produced when the environment begins to become unfavorable. Male *C. dubia* usually appear in autumn and those of *D. magna* in spring or autumn. *C. dubia* and *D. magna* reproduce via cyclic parthenogenesis with the male contributing genetic material only during certain times of the year. *D. pulex* may reproduce by either a cyclic or obligatory parthenogenesis. Daphnids produce ephippia, encased embryos that are resistant to harsh environmental conditions, during periods of sexual reproduction.

104. The life span of these species is highly variable, depending principally on environmental conditions. At 25°C, *C. dubia* and *D. magna* live about 30 days and 40 days, respectively. *D. magna* lives about 56 days at 20°C, whereas *C. dubia* and *D. pulex* live about 50 days at that temperature (Weber 1993). These daphnids produce about 10 eggs per clutch, which hatch into juveniles after about 38 h; the maximum egg production occurs at temperatures from 18°C to 25°C.

105. Availability, Culture, and Handling: All three species are readily available from commercial sources and are easily maintained in culture. Only 20–30 individuals are required to start a culture within a testing laboratory. Cultures should be maintained for at least two generations under the same conditions and feeding regimes that will be used during testing. Cultures can be maintained in natural waters, but Weber (1993) recommends "synthetic" water because it produces reliable results. Culture temperature is optimal at about 20°C. *D. magna* requires relatively hard water (160–180 mg/L $CaCO_3$), whereas *D. pulex* requires softer water (80–90 mg/L $CaCO_3$). Dissolved oxygen concentrations should be held above 5 mg/L. Cultures should be maintained carefully. The culture medium should be replaced three times per week and the cultures should be thinned when the populations exceed 200 individuals per 3 mL of culture water. Daphnids should be fed a YCT mixture and the alga *Selenastrum capricornutum*.

106. Strengths and Weaknesses: Daphnids offer many advantages to an EDC evaluation testing program. They are ecologically relevant, widely distributed, and are readily available via commercial suppliers. Cultures are easy to start and maintain. Daphnids are usually tested at intermediate temperatures (20°C). Acute and chronic protocols for testing daphnids are available and daphnids are actively used in many testing scenarios. Daphnids have been actively used in the evaluations of potential endocrine disrupting chemicals (Section 3.2). The primary weakness with using daphnids in endocrine disruption evaluations is that the asexually reproducing stage is typically the one used in testing because the presence of sexual reproduction is taken as an indication that culture conditions are less than ideal or that testing

methods are deficient (Olmstead and LeBlanc 2000). Parthenogenic reproduction results in lower genetic variability within the test population than would occur with a sexually reproducing taxon (Lagadic and Caquet 1998). This reduced variability would reduce the potential variation in response to stressors. However, the emphasis on only testing the parthenogenic phase for endocrine disruptive effects ignores the sexual reproduction phase, which is vital to daphnid population viability (Lagadic and Caquet 1998, Olmstead and LeBlanc 2000). Therefore, EDC impacts to daphnid populations may be severely underestimated. The species discussed above are temperate species and may not be feasible for use in tropical areas. Buratini et al. (2004) evaluated a tropical species, *Daphnia similis*, and found it to be similar in sensitivity to the two temperate *Daphnia* species. *D. similis* is easily cultured and may be more appropriate for use in evaluating potential EDCs in tropical waters that have low hardness.

4.2.3 Copepods

107. Natural History: Copepods comprise one of the most important groups of marine invertebrates in estuarine and marine systems whether planktonic or benthic. They form a vital link in water column food webs, feeding on smaller plankton and being consumed by larger predators, such as fish. In benthic systems they are often important grazers on microalgae, yet provide forage for young life stages of many fish. The life history of copepods involves morphologically distinct stages (nauplius and copepodite stages). Therefore, it is possible to test the effects of chemicals, including those that are potential endocrine disruptors, on the metamorphosis from naupliar to copepodite stages that might detect interference with processes regulated by ecdysteroids (Anderson et al. 2001).

108. Availability, Culture, and Handling: Some species of copepods, *Acartia tonsa* for example, may be available commercially. However, the species typically used in toxicity testing are easy to maintain in cultures so that sufficient animals for testing are always available. Some *Acartia tonsa* cultures have been maintained for at least 20 years (Anderson et al. 2001). Culture temperatures depend on the species involved. *A. tonsa* and *Tisbe battaglia*i are cultured at 20°C (Anderson et al 2001, Hutchinson et al. 1999a, 1999b), *Nitocra spinipes* at 22°C (Breitholtz et al. 2003), *Amphiascus tenuiremis* at 22°C, and *Tigriopus japonicus* at 25°C (Marcial et al. 2003). Copepods are typically fed unicellular algae, such as *Rhodomonas*, *Nanochloropsis*, and *Isochrysis*.

109. Strengths and Weaknesses: Copepods are ecologically relevant animals for use in the evaluation of EDCs. They are readily available and easy to maintain and handle. The test conducted by Anderson et al. was a 5-6 day subchronic test with a very simple and easy to measure endpoint, the proportion of larvae that metamorphose from nauplius to copepodite. *A. tonsa* was more sensitive than *Daphnia magna* to interruption of growth and molting. Chronic (full life-cycle) tests may be completed within a relatively short time, from 21 days to 25 days. Although only one formal chronic test protocol has been developed (ASTM E2317-04 with A. tenuiremis), many copepod species have been used in toxicity testing, including the evaluation of potential EDCs (Section 3.2). The procedures used for those experiments could be adapted for standardized testing worldwide. There is ongoing work to develop an OECD copepod test guideline; a draft is written and pre-validation activities have been completed.

4.2.4 Mysids

110. Several mysid species are considered below for their potential utility in EDC testing. For each, a discussion of natural history, availability, and culture and handling is offered, along with a summary paragraph on strengths and weaknesses of the species as test organism. Ingersoll et al. (1999) included several additional characteristics that are important considerations in the selection of test species. Some of the features, for example mode of reproduction and knowledge of endocrinology, do not allow for discrimination among candidate mysid species. [For several of the species in the section below, the name

of the researcher who described them is cited. If the species was originally described in a genus other than the one listed, the researcher's name is enclosed within parentheses.

Americamysis bahia (Molenock, 1969)

111. *Americamysis bahia* is a small mysid crustacean that occurs in coastal estuaries and embayments ranging from the Gulf of Mexico to Narragansett, Rhode Island (Price et al. 1994).

112. Natural History: *A. bahia* typically occurs in areas where the salinity is >15‰, but is more abundant in higher-salinity habitats. Molenock (1969) originally described the species as *Mysidopsis bahia*, but Price et al. (1994) transferred it to a new genus, *Americamysis*, during their taxonomic revision. The natural history of the species is well known; the following description is based on Weber (1993). Adults of *A. bahia* may reach almost 10 mm in total length, and females attain a larger size than males. Individuals become sexually mature in about 12 to 20 days, and the genders can be distinguished when the animals reach about 4 mm in total length, at which time the brood pouch typically has started development. At about 12 days, the female's ovaries begin to contain eggs, and the brood pouch is fully formed at about 15 days. The developing young are carried in the brood pouch for an additional 2 to 5 days, resulting in a life cycle of about 17 to 20 days. New broods may be produced about 4 to 7 days. Females produce an average of about 11 eggs per brood, and the number is directly related to female body length.

113. Availability, Culture, and Handling: *A. bahia* is cultured commercially by many laboratories located throughout the United States and therefore is readily available to testing laboratories. With only a few days' notice, commercial suppliers can ship <24-h-old mysids via overnight service, allowing testing laboratories time to acclimate the animals to test conditions. However, testing laboratories can also easily culture the species. The mysids can be raised in 80-L to 200-L aquaria provided with continuous flow-through or recirculation systems. The water temperature and salinity within the culture tanks are typically 24ºC to 26ºC and 20‰ to 30‰, respectively. The cultures are fed newly hatched brine shrimp (*Artemia* sp.). Several sources provide information on culturing this species (e.g., Lussier et al. 1988). Although a small species, individuals of *A. bahia* are relatively easily handled.

114. Strengths and Weaknesses: As a candidate test species, *A. bahia* has many strengths and few weaknesses. Its primary advantages include its widespread availability and ease of culture. Because animals can be obtained from commercial cultures, the likelihood of misidentifying the species is very low. Its relatively short generation time makes it desirable in life cycle testing. The species is widely used in toxicological testing, and appropriate test conditions are well known. Standardized life cycle test protocols have been developed (US EPA 1996; ASTM 1997) and applied (e.g., McKenney 1982, 1985, 1986, 1994; McKenney et al. 1991; McKenney and Celestial 1996) or evaluated (Lussier et al. 1999). Included among the standardized test protocols are many of those necessary to measure EDC-related endpoints; others appear in peer-reviewed publications (Ingersoll et al. 1999). McKenney (1998) synthesized the results of several of his earlier studies on the effects of chronic exposures to various pesticides on *A. bahia* and concluded that life-cycle endpoints (slowed juvenile growth and reduced production of young) were more sensitive than survival. More recently, studies showed that the results from toxicity tests, which included standard and multigenerational tests using *A. bahia,* could be used to extrapolate from laboratory to population effects (Kuhn et al. 2000; 2001). Verslycke et al. (2004a), in their review of the use of mysids for endocrine disruptor evaluations, favored *A. bahia* as a reasonable test species. One criticism of the widespread use of *A. bahia* in toxicological testing is that because it is a warm-temperate or subtropical species, it may not be ecologically relevant to colder-water materials testing.

Holmesimysis costata (Holmes, 1900)

115. *Holmesimysis costata*, previously referred to as *Acanthomysis sculpta*, is an ecologically important species that ranges from southern California to British Columbia (Hunt et al. 1997).

116. Natural History: *H. costata* is a dominant member of the plankton community living within the surface canopy of the giant kelp, *Macrocystis pyrifera* (Chapman et al. 1995) and is an important pelagic prey of the California gray whale, *Eschrichtius robustus* (Dunham and Duffus 2002). Adults may reach lengths of about 7 mm to 13 mm (Daly and Holmquist 1986), and females attain a larger size than males (Turpen et al. 1994). Sexual maturity occurs at about 42 days, at which time males, which are recognized by an extended fourth pleopod, can be distinguished from females, recognized by the developing brood pouch (Turpen et al. 1994). Young are released when the females are about 65 to 73 days old. Brood size among laboratory-cultured females averaged about 16 released juveniles per female; in contrast, field-collected females released substantially larger broods that averaged 27 young (Turpen et al. 1994). Brood size was directly related to female size. US EPA (2002a) reported that females may produce multiple broods during their 120-day life span. However, Turpen et al. (1994) also reported that all laboratory-reared females died before releasing a second brood of young.

117. Availability, Culture, and Handling: Field-collected animals are available from a few suppliers and are likely to be available year round, but the species is not cultured commercially (Turpen et al. 1994). Brood stock can be collected by sweeping a small-mesh net through the canopy of the giant kelp (US EPA 2002a). Field-collected mysids can be cultured in the laboratory, and guidelines for doing so have been established (Chapman et al. 1995, US EPA 2002a). However, brood stocks should be rejuvenated periodically by the addition of field-collected animals (Turpen et al. 1994). Culture tanks can range in volume from 4 L to 1000 L and should be provided with aeration and fronds from the giant kelp, *M. pyrifera* (Chapman et al. 1995, US EPA 2002a). *H. costata* is typically maintained and tested at temperatures that range from about 13ºC to 15ºC for animals collected north or south of Point Conception, CA, respectively (US EPA 2002a). Adults are fed newly hatched *Artemia,* whereas juveniles are fed *Artemia* supplemented with a small amount of ground fish-food flakes (e.g., Tetramin). The animals are easily handled by using a combination of small-mesh dip nets and pipettes to transfer them from culture tanks to test chambers.

118. Strengths and Weaknesses: The primary strengths of this species are its ecological relevance to northeast Pacific regional testing conditions, its relatively large brood sizes, and its ease of handling and maintenance. *H. costata* occurs in relatively cold waters and may serve as a coldwater alternative to *A. bahia*. The species has been used in several toxicological tests (e.g., Singer et al. 1998) and standardized test protocols for the species have been developed (Chapman et al. 1995, US EPA 2002a) and evaluated (Martin et al. 1989, Hunt et al. 1997). Martin et al. (1989) determined that *H. costata* had sufficient sensitivity to evaluate effluent toxicity and that the results were repeatable by different testing laboratories. The principal disadvantages inherent in using *H. costata* are its long generation time (~70 days) and the difficulty in raising multiple broods in the laboratory. The tests required to measure many EDC-related endpoints must be developed (Ingersoll et al. 1999) or are impractical because of the species long generation time and the difficulty in raising multiple broods. Because the original animals are field-collected, they must be identified carefully prior to their use in testing. Also, potential population-related differences in pollutant sensitivities should be assessed before comparing the results of tests performed with mysids from different populations.

Mysidopsis intii holmquist, 1957

119. *Mysidopsis intii* is an epibenthic species that occurs in the eastern Pacific from South America to the southern California coast of the United States (Price et al. 1994; Langdon et al. 1996).

120. Natural History: *M. intii* has only recently been reported from the United States (off Los Angeles), but it could be more widespread (Langdon et al. 1996). *M. intii* is a relatively small species that attains body length of about 6 mm to 7 mm. The genders can be distinguished at about 9 to10 days after hatching. Eggs enter the brood pouch at about 13 days, and juveniles are released at about Day 20 (Langdon et al. 1996).

121. Availability, Culture, and Handling: Animals to be used in testing must be obtained from field collections, because there is no commercial culture of the species. Individuals of *M. intii* are collected by using an epibenthic sled, and the wild-caught animals are then used to establish breeding stocks in the laboratory. The species is easily cultured in the laboratory in 40-L to 90-L tanks continuously supplied with flowing, filtered seawater. Langdon et al. (1996) determined that the optimal temperature for high juvenile production is 20ºC. To ensure high reproductive output, adults should be fed recently hatched *Artemia* and adult copepods, *Tigriopus californicus* (Kreeger et al. 1991; Langdon et al. 1996). The *Artemia* diet can be enriched with fatty acid supplements, and the mysid cultures provided with fatty acid boosters (UCSC 1998). Separation of juvenile mysids from the adult cultures is easily accomplished by using light to attract them through a 1-mm-mesh divider into an isolation chamber (Langdon et al. 1996).

122. Strengths and Weaknesses: *M. intii* represents an indigenous, ecologically-relevant species for testing contaminants that could negatively affect northeast Pacific coast ecosystems. The life cycle of *M. intii* is much shorter (~20 days) than that of *H. costata* (~70 days), the other Pacific coast species commonly used in toxicity testing. The US EPA sponsored the development of a 7-day toxicity test protocol (Langdon et al. 1996) that has been applied (UCSC 1998) and evaluated (Harmon and Langdon 1996). *M. intii* was recently used in a series of tests examining acute and chronic effects of nickel on three species of marine organisms (Hunt et al. 2002). The chronic test was a 28-d full life-cycle test that included survival, growth (as the change in length and weight), and two reproductive endpoints (the percentage of females carrying eggs or juveniles in the brood sac, and the number of live juveniles produced). However, during this test no juveniles were released and the number of gravid females was low in the test controls. Hunt et al. did not discuss possible explanations for the reproductive failures. The primary disadvantages associated with using *M. intii* is the lack of available commercial culture and that testing protocols to measure EDC-related endpoints need to be developed. Regardless of source of test animals, *M. intii* individuals used in testing must be identified carefully prior to their use in tests. Further, the requirement to supplement an *Artemia* diet with copepods (*T. californicus*), which initially must be field collected, could be an impediment to the use of *M. intii* by some laboratories. Finally, potential population-related differences in pollutant sensitivities should be assessed before comparing the results of tests performed on different populations.

Neomysis integer (Leach, 1814)

123. *Neomysis integer* is found throughout northern Europe (Mees et al. 1994) and has been suggested as an appropriate species for use in European toxicity testing programs (Roast et al. 1998, 2000b).

124. Natural History: *N. integer* is a relatively large, hyperbenthic species that occurs in relatively low-salinity portions of estuaries (Roast et al. 2001). Females may attain a standard length of about 18 mm (measured from the base of the eyestalk to the end of the last abdominal segment); males are smaller (Mees et al. 1994). Brood size is strongly correlated with the size of the female: the number of larvae per brood extends to about 80 individuals for females of 16 mm or more in length (Mees et al. 1994). Winkler & Greve (2002) determined that individuals matured in about 45 d after hatching at 15°C, but at about 3.5 months at 10°C; the total generation time was about 69 d at 15°C.

125. Availability, Culture, and Handling: Because it is the dominant mysid inhabiting northern European estuaries (Mees et al. 1994), it is readily available, but animals to be used in testing must be field

collected. It is not cultured commercially, but wild-collected animals are easily maintained in the laboratory.

126. Strengths and Weaknesses: The principal strength of this species for use in toxicity testing is that it is a common and ecologically important component of European estuaries. It is also a very well-studied species, ecologically, physiologically, and toxicologically. However, it is not commercially cultured and all animals to be used in tests must be collected from estuaries and raised in the testing laboratories. *N. integer* can be tested at relatively cool temperatures of about 15°C, although it is probably not a useful species for testing at colder temperatures (~10°C) because of the much longer generation time at cold temperatures. Testing to measure potential EDC-related endpoints and studies addressing some aspects of the endocrinology and of this species, and its potential utility in EDC testing, has recently been completed. Verslycke and Janssen (2002) developed an indicator, the CEA, that could be used to detect changes to energy metabolism of *N. integer* in response to environmental stressors and tested the effects of tributyltin chloride on the CEA (Verslycke et al. 2002, 2003a). Verslycke et al. (2002) found that *N. integer* produced 11 monohydroxy testosterone metabolites and two nonpolar metabolites (androstenedione and dihydrotestosterone). They also found the anabolic steroid β-boldenone, which had not been previously reported in invertebrates. The function of the steroids in mysids is not clear. Verslycke et al. (2002) did not detect the vertebrate estrogen 17β-estradiol in *N. integer*. Biotransformation experiments conducted by Verslycke et al. (2002) revealed that mysids have a complex steroid hydroxylase system comprised of several P450 isozymes. Alteration of P450 activity in mysids could be used as an endpoint in EDC testing. Verslycke et al. (2003b) tested the effects of TBTCl on testosterone metabolism in *N. integer* and found non-polar and polar metabolite induction at the lowest TBTCl concentration (10 ng/L) tested, but there were no significant differences from controls at higher concentrations. TBTCl had no effect on the elimination of testosterone by glucose conjugation, in part because of high within-treatment variability. Testosterone elimination by sulfate conjugation was significantly lower at high TBTCl concentrations. [Verslycke et al. calculated a metabolic androgenation ratio (the ratio of the oxido-reduced products to the hydroxylated plus conjugated products), which has been used to interpret the total effect of a chemical on testosterone metabolism, and found no significant treatment-related differences from the control.] Despite this, they contended that the ratio could be used to summarize the effect of TBTCl, which was an increase in the ratio at the two lowest concentrations. What was also clear in the data, but not discussed, was the very high variability in the ratio within treatments, especially within the controls. Control variability, as indicated by coefficient of variation values of almost 100%, was very high, which calls into question the general utility of the metric in evaluating endocrine disruption.]

127. Gorokhova (2002) stated that to understand growth of crustaceans (which is a potential EDC endpoint) it is imperative to understand the molt staging of the organism. Gorokhova determined that the duration of the molt cycle of *N. integer* was about 9 days and that the effects of salinity and temperature on molt staging differed according to the food supply.

128. These animals must be identified carefully prior to testing. Also, potential population-related differences in pollutant sensitivities should be assessed before comparing the results of tests performed on different populations.

Other mysid species

129. A few other species of *Americamysis*, including *A. almyra* (Bowman 1964) and *A. bigelowi* (W. Tattersall 1926), have been used in toxicity testing or related studies. *A. almyra* is closely related to *A. bahia*, and the two species have similar geographic distributions; however, *A. almyra* inhabits less saline waters (Price et al. 1994). The reproductive biology including brood size and generation time of the species is very similar to that of *A. bahia* (reviewed in Reitsema and Neff 1980). The species is amenable to laboratory culture. Recirculation (Reitsema and Neff 1980) and static (Domingues et al. 1998, 1999)

culture systems have been developed. *A. almyra* can be maintained on a diet of *Artemia* nauplii (Domingues et al. 2001a, 2001b). *A. bigelowi* is also biologically similar to *A. bahia* and to *A. almyra*. It occurs along the east coast of the United States from Massachusetts to Florida (Price et al. 1994). Although Gentile et al. (1982) found it to be suitable for use in toxicology testing; *A. bigelowi* has not received widespread attention as a test species.

130. *Neomysis mercedis* Holmes 1897, an ecologically important Pacific coast species, was advocated as an acute toxicity test organism appropriate for estuarine waters having low salinities, ranging from 1‰ to 3 ‰ (Brandt et al. 1993). The suggested temperature range for testing with this species is 16°C to 19°C (Brandt et al. 1993); thus it could serve as a cool water alternative to *A. bahia*. It occurs in freshwater and brackish waters from California to southern Alaska (Daly and Holmquist 1986) and recently has been used in toxicity testing (Farrell et al. 1998a, 1998b; Hunt et al. 1999, 2002). *N. mercedis* is an important predator on *Daphnia* in a freshwater lake (Lake Washington, Washington), where its consumption is enough to control *Daphnia* populations (Chigbu 2004). Brandt et al. (1993) found that *N. mercedis* was similar to *A. bahia* in sensitivity to several environmental contaminants. A standardized acute toxicity testing protocol has been developed for *N. mercedis* (ASTM 1997). *N. mercedis* is not cultured commercially, but can be reared in the laboratory (Brandt et al. 1993). *N. mercedis* has a relatively long generation time of about three to four months (Brandt et al. 1993). The tests required to measure many EDC-related endpoints must be developed (Ingersoll et al. 1999) or are impractical because of the species' long generation time.

131. *Neomysis americana* (S.I. Smith, 1874) is a western Atlantic species that occurs on sandy bottoms at depths of 0 to –240 m from Florida to Newfoundland, and also South America (Anderson et al. 2004). *N. americana* plays a dual role in marine food webs, being an important item in the diets of several species of demersal fish (Steimle et al. 2000) and a significant predator on zooplankton (Winkler et al. 2003). The species was used in some toxicity tests in the late 1970s, early 1980s, but has been used very little. The US EPA lists *N. americana* as an alternative species for an acute effluent testing protocol (US EPA 2002a), but not for chronic effluent testing (US EPA 2002b). Typical test temperatures are 20°C and 25°C and the typical salinity range is from 10‰ to 32‰ (US EPA 2002a).

132. *Praunus flexuosus* (Müller 1776) is one of the two predominant mysid species in northern European waters, particularly in shallow water in the outer Elbe Estuary, where it is predominant in summer (*N. integer* is other abundant species) (Winkler and Greve 2002). *P. flexuosus* is tolerant of a wide range of salinities and temperatures. It is not commercially cultured and must be collected from coastal waters before testing. Two studies by Garnacho and coworkers have highlighted one of the important caveats that must be recognized when using wild-collected animals for EDC evaluations. Garnacho et al. (2000, 2001) examined the effects of copper on survival and the metabolism of *P. flexuosus*. The mysids were fed <48-h old *Artemia* nauplii during the holding period and daily during testing. Testing was conducted with water at a salinity of 33‰ and temperatures of 10°C and 20°C. Water and test solutions were renewed every 48 h. Copper was found to be toxic to the mysids collected and tested during the summer, whereas it was not toxic to mysids collected and tested during the winter (Garnacho et al. 2000). Garnacho et al. found that the O:N ratio varied seasonally, being 2.5 times greater in winter than in spring. Metabolism of species is protein based all year. Garnacho et al. also found that the O:N ratio decreased quickly (24 h) at the highest copper dose and decreased in all doses by 10 days. The response in summer was faster than that in winter. The shift in O:N ratio reflects a greater metabolic reliance on protein. The faster response in summer occurs when metabolism is already more dependent on protein. The observed response to copper differs from other mysid responses to stress. Garnacho et al. concluded that O:N ratio changes appears to be a sensitive early indicator of stress in mysids. However, one significant finding from the two studies was that the effects of a particular toxicant or stressor on animals that are wild-collected might vary importantly depending on the season during which the animals were collected. Winkler and Greve (2002) determined that *P. flexuosus* did not mature at a test temperature of 10°C, but

did grow to large size (20 mm). At 15°C, individuals matured at about 3.5 months after hatching and reached a mean size of 16 to 18 mm. The mean incubation time within the marsupium was about 23 d at 15°C, and the total generation time (from egg to mature individual) was about 133 d. Despite its ecological importance, *P. flexuosus* would not be a useful colder water test animal, and its long generation time makes it undesirable for EDC evaluations.

133. *Tenagomysis novaezealandiae* Thomson 1900 is found on New Zealand's North and South Islands (Nipper and Williams 1997). The species is abundant and widely occurring. Although it is not available through commercial culture, field-collected animals can be maintained in the laboratory. The species' life cycle lasts about four weeks and laboratory populations can be reproductively active year-round (Nipper and Williams 1997). Mysids can be collected by using hand dip nets and are cultured in the laboratory at a temperature of 20°C, a salinity of 34 ‰, and a light:dark photoperiod of 16:8 hours. Mysids are held at a density of 10–20 individuals/L and are fed newly hatched brine shrimp nauplii daily. Nipper and Williams (1997) conducted several experiments designed to determine the appropriate physical conditions and food regimen for holding and testing the species. One of the more interesting findings from these experiments was that survival was higher when tests were conducted in complete darkness compared to the 16:8-h light:dark cycle. Nipper and Williams attributed this to the typical vertical migration habits of mysids, which involves migration up in the water column at night and resting in dark areas near the bottom during the day. Nipper and Williams (1997) concluded that *T. novaezealandiae* is suitable for use in toxicity testing and can be cultured for year-round testing.

4.2.5 Decapods (shrimp, crabs, crayfish, lobsters)

134. Decapods probably have been the subjects of more endocrine function and possible EDC effects-related testing than any other crustacean group (Fingerman et al. 1998, Ingersoll et al. 1999). Decapods are particularly appealing to EDC studies because much is known about their endocrine systems and their free-swimming larvae are likely to be susceptible to JH analogs and other Insect Growth Regulators (IGRs) produced to control insects (McKenney 1999).

135. Two species, the mud crab (*Rhithropanopeus harrisii*) and the grass shrimp (*Palaemonetes pugio*), have been the primary subjects for many of the endocrine studies and have been advocated as being potentially useful in studies of EDCs (McKenney 1999). Several studies have investigated the effects of EDCs on crustacean life cycles using decapod larvae as the test organisms (Section 3.2). Most of the recent work on the impacts of various EDCs on decapods has centered on the grass shrimp *Palaemonetes pugio* and the mud crab *Rhithropanopeus harrisii*, which, therefore, are the focus of this section.

136. Natural History: The grass shrimp, *Palaemonetes pugio*, is widespread in estuaries from Nova Scotia to Texas (Williams 1984) and has been used considerably in bioassays. Grass shrimp feed on bacteria-laden benthic detritus and are an extremely important component of energetic flux pathways in estuaries (studies summarized in Williams 1984). Gravid females are available from late spring to late summer. Grass shrimp usually mature about six months after hatching, although maturation may occur quicker where water temperatures are warm (Williams 1984, Volz et al. 2002). The mud crab, *Rhithropanopeus harrisii*, occurs in estuaries from the southwestern Gulf of St. Lawrence, Canada to Veracruz, Mexico and has been introduced into parts of Europe and the U.S. west coast (Williams 1984). Mud crabs tolerate a wide range of salinities and temperatures. Muds crabs are omnivorous, feeding on a variety of aquatic plants, detritus, and other estuarine animals. Gravid females typically can be collected during the summer months. Mud crab larvae, rather than adults, are typically used in toxicity studies.

137. Availability, Culture, and Handling: Grass shrimp are available from commercial suppliers and can be maintained in culture by testing laboratories. Mud crabs must be collected from the field before testing. Grass shrimp can be held at a wide range of conditions with temperatures ranging from 18°C to

25°C and salinities ranging from 20‰ to 28‰. Ovigerous females are held in the laboratory until the eggs hatch. Larvae, which are used in toxicity testing, can be cultured at the above conditions in the laboratory. Many studies involving the mud crab use the larval stages rather than adults. Ovigerous females can be collected and held in cultures until the larvae, which are relatively easy to raise, are released. Larvae can be cultured at a water temperature of about 25°C and a salinity of about 20‰. Larvae of both species are fed brine shrimp (*Artemia*) nauplii daily.

138. Strengths/Weaknesses: The principal strength that both species offer an EDC evaluation program is that the knowledge of decapod endocrine systems is relatively well-developed. Both are common animals in estuaries of the U.S. Gulf and East Coasts. One species, the grass shrimp, is commercially available. Both are amenable to individual, focused studies of the specific impacts of EDCs on various endocrine system components and, thus, are important species to study. However, neither is advantageous for use in an overall EDC screening program, primarily because the long generation time precludes relatively rapid multigenerational studies. Additionally, the mud crab must be collected from the field and would be difficult for many testing laboratories to obtain.

4.3 NON-ARTHROPOD SPECIES

139. Estuarine and marine communities are comprised of taxa from many phyla other than the Arthropoda. Some taxa, especially polychaete worms, are of very high ecological importance and are probably very susceptible the EDCs. Many frequently are used for various types of regulatory toxicity testing. However, the endocrine systems of most have not been studied sufficiently, nor have had adequate protocols been developed, for these taxa to be included in an EDC evaluation program. Nonetheless, three of these groups of taxa are briefly reviewed in this section.

140. Polychaetes: Polychaete worms comprise an important component of most estuarine and marine ecosystems. Primarily infaunal sediment dwellers, polychaetes contribute significantly to sediment bioirrigation and occupy a key part of marine food webs. They represent an ecologically significant group on which to evaluate the effects of potential EDCs. Several species of polychaetes are widely used in sediment toxicity testing. Polychaete (e.g., *Nereis*) bioaccumulation assays are one of the primary tools used in evaluating the suitability of dredged material for disposal into U.S. coastal waters (US EPA/USACE 1998 ITM). A general toxicity testing protocol for has been developed for several species of nereid polychaete worms, including a chronic survival and growth assay for *Neanthes arenaceodentata* (ASTM 2000) and a fertilization/embryo-larval development assay for *Platynereis dumerilii* (Hutchinson et al. 1995). *P. dumerilii* has been widely used in Europe, especially in genotoxicity studies (e.g., Haggar et al. 2002) and some aspects of its reproductive endocrinology have been studied (review in Andries 2001). Several age categories of *N. arenaceodentata* are available from a commercial supplier. Several breeding cultures of *P. dumerilii* have been established at European laboratories (e.g., the European Molecular Biology Laboratory in Heidelberg, Germany). The species can be bred continuously in the laboratory (Fischer and Dorresteijn 2004) and may eventually be a good candidate for EDC evaluations. The prime weakness in using polychaetes for EDC evaluations probably is the lack of understanding of functional roles for hormones in polychaetes (Andries 2001).

141. Mollusks: Mollusks may seem an obvious choice for use in EC evaluations because imposex is frequently offered as the best example of endocrine disruption in invertebrates (Section 3.2). Bivalve (e.g., *Macoma*) bioaccumulation studies are important in contaminated-sediment evaluations (US EPA/USACE 1998), general chronic assays have not been developed. Endocrine disruption in marine gastropods has been studied considerably, almost exclusively with respect to the induction and incidence of imposex. However, the effects, including reproductive endpoints, of some endocrine disrupting chemicals on the hermaphroditic freshwater snail *Lymnaea stagnalis* have been studied (Czech et al. 2001, Coeurdassier et al. 2004). *Lymnaea peregra* was included in a multi-species multi-generation, whole life-cycle testing

protocol developed by Sánchez and Tarazona (2002). However, at this point suitable chronic protocols for estuarine and marine mollusks have not been developed that could be adapted for a chronic reproductive assay.

142. Echinoderms: Echinoderms are often included in some regulatory testing protocols, particularly those involving effluent discharges or the effects of the suspended particulate phase of sediments, but these focus on acute effects on embryonic and larval development (e.g., US EPA 2002b, 2002c). Some echinoderms have been used in studies of specific EDCs (Section 3.2), but these do not involve chronic procedures or reproductive endpoints. Echinoderms would be of interest to an EDC evaluation program because they are known to have steroids that also occur in mammals (LeBlanc et al. 1999). Currently, echinoderms do not seem well-suitable to multi-generational EDC evaluations.

5.0 SELECTION OF AN APPROPRIATE TEST SPECIES AND PROTOCOLS

143. Several characteristics should be considered in determining the most appropriate species for use in EDC-related testing. Nimmo and Hamaker (1982) and Roast et al. (1998) suggested that the test organism should be ecologically relevant, and sensitive to contaminants. Because it must be available as needed for testing, it must be abundant and easily collected, or amenable to laboratory culture. Its diet should be well understood and easy to provide in the laboratory, therefore allowing it to be well-adapted to laboratory conditions and reducing the need for extensive acclimation periods. The ideal test organism should have a short, relatively simple life cycle that allows for the testing of successive generations.

144. Ingersoll et al. (1999) included several additional characteristics that are important considerations in the selection of test species. These characteristics include the species' mode of reproduction and knowledge of their endocrinology. Species that reproduce parthenogenetically may produce a test population within which genetic variability is relatively low compared to species that reproduce sexually. High genetic variability may yield highly variable responses to a stressor within a test population (Lagadic and Caquet 1998), which may make it difficult to detect impacts other than those that are substantial. However, using parthenogenetically reproducing animals means that important processes related to sexual reproduction (e.g., gametogenesis) are not being evaluated (Lagadic and Caquet 1998, Olmstead and LeBlanc 2000). Knowledge of the endocrinology of a candidate species is important to the selection of appropriate test endpoints and the interpretation of the test results (Segner et al. 2003). Also important to selecting a candidate species for use in the multigenerational EDC testing program are the ability to culture the species in the laboratory (with a strong probability that transgenerational testing is possible); relatively short generation time, allowing for full life-cycle testing; size (larger animals provide more tissue for measuring hormone titers, but usually have longer generation times); and the availability of standard (consensus-based) testing methods, including whether or not new methods must be developed to measure EDC-relevant endpoints.

145. Recent studies of the genetic variability within a common and widespread species emphasizes the need to document the test species identity and the source from which the animals originated. Several studies have shown that the freshwater amphipod *Hyalella azteca* is a species complex comprised of at least a dozen species and that different genotypes may vary in their response to the same stressors (discussed in detail in section 4.2.1). The identity of test organisms should be verified and documented through a voucher collection, as described by Huber (1998) that is maintained by the testing laboratory.

5.1 CRUSTACEANS AS REPRESENTATIVE AQUATIC INVERTEBRATES

146. Many insecticides are considered to be EDCs, because they are specifically formulated to attack insect endocrine systems, affecting in particular the systems that are involved in molting and larval metamorphosis (Oberdörster and Cheek 2001). Most of these insecticides are JH analogs (Oberdörster and Cheek 2001) or ecdysteroids. Although insects and crustaceans represent two classes within Phylum Arthropoda and consequently exhibit many similarities as well as differences, several recent studies showed that insecticides formulated as JH analogs adversely affect crustacean larvae by disrupting molting and metamorphosis (e.g., see McKenney and Celestial 1996, which focuses on mysids). Crustaceans probably do not synthesize JH (LeBlanc et al. 1999); however, they do produce methyl farnesoate in the mandibular gland, and it is likely a natural JH analog. It is known to be involved in crustacean reproduction (LeBlanc et al. 1999) and development (Borst et al. 1987), but its specific role is uncertain.

147. Chronic testing protocols for freshwater and estuarine or marine arthropod species have been developed by several regulatory agencies. Examples of protocols developed by national regulatory

agencies and by OECD on daphnids, on chironomids, on other sediment dwelling invertebrates, on mysids and on copepods are highlighted below. Also, current OECD or draft OECD guidelines for each group of species are presented in the annex section.

5.1.1 Mysids as representative crustaceans

148. Mysid crustaceans are distributed from 80 ºN to 80 ºS and occur in most aquatic environments, including brackish, freshwater, and marine (e.g., Mauchline 1980). In some habitats, particularly coastal temperate waters, mysids are very abundant and are very important in freshwater, estuarine, and marine food webs. Mysids diets include consumption of detritus, phytoplankton, and zooplankton. They may be abundant enough to control population densities of some prey (Chigbu 2004). Stomach-content analysis revealed that mysids are a staple food for striped bass, the tidewater silverside, and several species of flounder (Gentile et al. 1983). Many estuarine mysids are also hyperbenthic (Roast et al. 1998) and make diurnal migrations into the water column (Dauvin et al. 1994). For these reasons, mysids could serve as sensitive indicator species to monitor the effects of EDCs through exposure to either the water column or sediment or through dietary uptake.

149. Mysids, despite their superficial resemblance to decapod shrimp, have been considered more closely related to amphipods and isopods (Brusca and Brusca 1990). However, recent studies of 28S rDNA sequences among several malacostracan orders indicated that mysids (Suborder Mysida) are more closely related to krill (Euphausiacea) than to amphipods or isopods (Jarman et al. 2000). This proposed phylogeny has not been accepted universally (Richter and Scholtz 2001). Regardless of phylogenetic relationships, mysids, amphipods, and isopods are characterized in part by the retention of developing young in a marsupial brood pouch. All three taxa would be good candidates for toxicological testing, and amphipods and mysids are routinely used. However, for EDC testing, especially for life cycle tests, mysids offer clear advantages over amphipods. Most marine amphipods used in toxicological testing must be collected from their natural habitats just prior to use in tests. Although they can be held for a few weeks prior to testing, they generally are not cultured for tests. Currently, only one marine amphipod, *Leptocheirus plumulosus*, has been cultured successfully and used for growth and reproduction tests (US EPA 2001). Conversely, several mysid species have been cultured in the laboratory and used in such life cycle tests.

150. Nimmo and Hamaker (1982) advocated the use of mysids in toxicity testing, including acute and chronic life-cycle testing. Nimmo and Hamaker compared acute and chronic toxicity data for a variety of taxa and concluded that mysids were more sensitive than most of the taxa, including grass shrimp, an estuarine fish, and 17 freshwater species. Nimmo and Hamaker foresaw a role for mysids in the evaluation of the effects of pesticides and metals on successive generations, in part because the life cycle of mysids is short. This role is being fulfilled by the inclusion of mysids in an EDC evaluation program. Mysids are currently also used in routine toxicity tests to examine the potential toxicity of marine sediment (Carr et al. 1998; Cripe et al. 2000). It has been documented (Cripe et al. 2000) that the mysids were observed to collect sediment, manipulate it at the mouth region, and drop it. This suggests that mysids could be used to test sediment suspected of containing EDCs. Further, Verslycke et al. (2004a) evaluated the potential for using mysids in an endocrine disruption evaluation program. They reviewed aspects of the biology and ecology of mysids in the context of this potential use and summarized the literature on toxicity studies done using mysids. Their review also included a discussion of many endpoints that may be useful when using mysids to test for endocrine disruption. Verslycke et al. (2004a) concluded that mysids have the ecological relevance and sensitivity to stressors required of a taxon that would be suitable for evaluation of endocrine disruption in marine and estuarine invertebrates and could serve as a surrogate for other crustacean species.

5.1.1.1 Protocols using mysid species

151. Specific guidance for conducting short-term toxicity tests with species other than *A. bahia* has been published. It is possible that these protocols can be modified to allow longer life cycle testing.

152. *Holmesimysis costata*.—Chapman et al. (1995) described a 7-day test protocol designed to measure growth and survival in tests using the west coast mysid species, *H. costata*. In addition to recommended test conditions, guidance in culturing the animals and analyzing the data are presented. The protocol describes the ecological and culture requirements for *H. costata;* this information could be used to modify the ASTM and OPPTS protocols described above to allow longer life cycle testing. The test protocol was evaluated by means of a series of intra- and interlaboratory comparisons (Hunt et al. 1997), which concluded that this test had sufficient sensitivity and precision to make it useful in testing possible contaminant impacts.

153. *Mysidopsis intii*.—A short-term toxicity test protocol for a west coast species, *M. intii,* was developed with support from the US EPA (Langdon et al. 1996). The protocol concisely describes the test conditions required to conduct a 7-day toxicity test to measure survival and growth of this species. Initial test development was performed using zinc sulfate as the toxicant. The test protocol was evaluated by means of an interlaboratory comparison that employed sodium dodecyl sulfate as the toxicant (Harmon and Langdon 1996). Harmon and Langdon (1996) also compared *M. intii* test with those using *A. bahia* and *H. costata,* and reported that its sensitivity was equal to that of the *A. bahia* test, but that it was lower than that of the *H. costata* test.

154. A proposed guideline submitted to OECD for consideration for further development, validation, and acceptance has been developed (OECD 2004a, see also Annex 1). The objective of a mysid two-generation reproductive and developmental toxicity test is to provide the most precise and accurate estimate of toxicity associated with endocrine disruption and reproductive fitness for an identified potential EDC. The results of the Tier 2 testing should be conclusive in documenting a discernible cause-and-effect relationship of chemical exposure to measurable manifestation in the test organisms. The test protocol will be designed to:

- Determine whether effects are a primary or secondary disturbance of endocrine function
- Establish exposure/concentrations/timing and effects relationships
- Be sensitive and specific
- Assess relevant endpoints
- Include a dose range for full characterization of effects (EDSTAC 1998).

155. The test is an extension of previous mysid life-cycle test procedures produced by ASTM (ASTM 1997; see Table 3) and US EPA (Nimmo et al. 1977, 1978, McKenney 1986, 1998; see Table 3). Basic principles of experimental design from these two protocols must however be followed. The protocol provides general guidance for conducting a two-generation toxicity test in which the parent generation (F0) is exposed to the toxicant and the parent and first offspring (F1) generations are monitored for 7 days after the mean date of release of the second brood by the control treatment parent generation (F0). The guideline also allows for the exposure of the F1 generation to the test substance. The guideline is applicable to *Americamysis bahia*, but could be modified to suit other mysid species. The two-generation test is initiated when healthy < 24-h-old mysid juveniles are placed randomly into replicate test chambers. These original juveniles comprise the parent (F0) mysid stock for the test. Development, sexual maturation, reproduction, and growth are observed in the F0 and F1 generations. The assay is conducted

with at least five toxicant concentration treatments and appropriate control treatments (typically a 0% concentration and a solvent control, if one was used to deliver the toxicant to the test treatments). The highest exposure concentration should be equal to the lowest concentration that caused adverse effects in the acute test or 1/10 the LC_{50} (EC_{50}). Toxicant and control treatments are delivered to the mysids in water, which is continuously or intermittently delivered to the test chambers via a proportional diluter system or infusion pumps. The specific exposure duration will vary, but is at least 7 days longer than the median second-brood-release date by the original parent mysids in the control treatments. During the test, chambers are examined for mysid mortality, the presences of molted exuviae, the presence of ovigerous females, and the release of young, all of which are recorded. Young from the first brood (= F1') release by the parent stock are held for four days, after which they are counted and measured. The parent stock is allowed to produce a second brood (= F1"), after which the parent mysids are counted and measured. The F1" mysids are exposed to the test materials and maintained until their first brood is released (= F2), after which the F1" mysids are counted and measured. The test is terminated with the release of the F2 mysids, which are counted. Surviving organisms may be analyzed biochemically, as appropriate.

5.1.2 Daphnia as representative crustaceans

156. *Daphnia* is a genus of small cladoceran crustaceans, most species of which inhabit lakes and ponds as a major constituent of zooplankton communities. Using pairs of legs with setae (hairs), they feed on protozoa, algae, bacteria and organic detritus. They are often positioned as key species in aquatic ecosystems, both as grazers, which feeds on primary producers (algae), and as a major part of the diet of fish and invertebrate predators. Most cladocerans exhibit cyclical parthenogenesis with both asexual and sexual reproduction existing in the life cycle. They produce females by parthenogenesis as long as environmental conditions are favorable. Males appear and sexual reproduction occurs, triggered by the change in environmental conditions, such as day length, food concentration and population density (Hobaek and Larsson 1990, Kleiven et al. 1992). Males may also be produced parthenogenetically and are genetically identical to females as a consequence of the environmental sex determination (Hebert 1987). Sexual reproduction occurs by the mating between males and females having haploid eggs. Fertilized eggs develop into resting eggs, which are enclosed with protective membranes and are resistant to desiccation and freezing. The production of resting eggs enables daphnids to be dispersed to other lakes or ponds by their attachment to waterfowl and aquatic insects. The eggs can survive for years preserved in bottom mud as “egg banks” analogous to the seed banks of many terrestrial plants (Marcus et al. 1994).

157. With its short generation time and ease of handling in the laboratory, daphnids have become one of the most popular organisms in ecotoxicological research and testing. There are several methods for the regulatory reproductive toxicity testing of daphnids (e.g., Environment Canada 1992, OECD 1998a, US EPA 2002b, National Institute of Technology and Evaluation, Japan 2004, see also below for details). It might be claimed that the particular nature of the reproductive system of cladocerans and the lack of pertinent endocrinological information may render them unsuitable organisms for testing the effects of potential endocrine disrupting chemicals on crustaceans in general. Recent studies, however, revealed that exposure to juvenile hormones in insects and crustaceans, or to pesticides formulated as juvenile hormone mimics, induce *D. magna* to produce male neonates, and that juvenile hormones and their analogs are involved in sex determination (Olmstead and LeBlanc 2002, 2003, Tatarazako et al. 2003, Oda et al. 2005b). Ten chemicals are known to induce the production of male neonates in *D. magna* (Tatarazako et al. 2003, Oda et al. 2005b). The same phenomenon has been reported for other cladoceran taxonomic groups (Oda et al. 2005a, Kim et al. 2006). Although the mechanism is not yet clear and the evidence is only circumstantial, it is suggested that juvenile hormones are involved in the determination of offspring sex by mothers or in the shift of reproductive mode from parthenogenesis to sexual reproduction in cladocerans that exhibit cyclical parthenogenesis. Ecdysteroids are also known to inhibit ecdysis or to cause an abnormal ecdysis in *D. magna* (Baldwin et al. 2001). This empirical evidence supports the

suitability of using daphnia methods for testing chemicals with possible endocrine disrupting effects on crustaceans.

5.1.2.1. Protocols using daphnid species

158. US EPA (2002a) developed a chronic protocol for use in the National Pollutant Discharge Elimination System (NPDES) Permits Program to identify effluents and receiving waters containing toxic materials in chronically toxic concentrations. The method includes chronic testing procedures for a daphnid (*Ceriodaphnia dubia*) that, although not designed specifically for EDC testing, could be modified use in such a program. The method also may be adapted for other daphnid species, such as *Daphnia major* and *D. pulex*. Test conditions and endpoints from the US EPA protocol are presented in Table 5. The test is continued until 60% of the control animals produce three broods of young. If this does not occur by 8 days, the test must be repeated. Environment Canada (1992) also has developed a similar chronic protocol for *C. dubia* that includes reproductive endpoints.

159. OECD revised its original daphnid test procedure (TG 201), separating the acute and chronic components into two guideline documents. The new chronic guideline (TG 211; see Table 4), which was written specifically to test *Daphnia magna*, was adopted in September 1998 (OECD 1998). OECD recommends that the data from the acute test be available for use in determining the appropriate test substance concentrations for the chronic evaluation. Newly hatched daphnids (<24-h old) are exposed to the test substance for 21 days. The primary endpoint measured is the total number of living young produced per adult daphnid that is still alive at the termination of the test. Any young produced by adults that die during the test are not included in the endpoint calculation. Secondary endpoints may include adult survival, growth, and the time to first brood. Japan (OECD 2003b) has proposed a draft enhancement to TG 211 that includes endpoints specifically intended to detect endocrine disruption in *Daphnia*. These are the offspring sex ratio, which is used as an endpoint for juvenile hormone-like chemicals; and molt inhibition, which is used as an endpoint for molting hormone-like chemicals. These endpoints are based on data reported by Baldwin et al. (2001), Olmstead and LeBlanc (2002, 2003), and Tatarazako et al. (2003). See Annex 2 for the OECD technical guidance Document No 211.

160. ASTM reapproved its standard guide for conducting life-cycle assays using *Daphnia magna* (ASTM 2004a). Newly hatched daphnids (<24-h old) are exposed to the test substance for 21 days. Biological data collected include mortality (recorded daily), the number of young produced (determined three times per week), size (dry weight) of the first-generation individuals still living at the end of the test, time to first reproduction, and behavioral abnormalities. Second-generation daphnid responses, such as survival, development, and behavior, may be obtained by observing these daphnids for an additional four days or more.

5.1.3 Harpacticoid and calanoid copepods as representative crustaceans

161. Copepods occur widely in almost every marine, brackish and freshwater ecosystem. They represent important prey items for the larvae and juveniles of many fish and larger invertebrates, and are increasingly used as a live food source in aquaculture. A number of copepod species (predominantly marine and brackish water species) have been used for many years to evaluate the acute and chronic toxicity of single chemicals and complex mixtures in water and sediments (Chandler and Green 2001). Copepod development is characterized by the accomplishment of eleven molt cycles. All together the juvenile copepods pass through six naupliar and five copepodite stages before turning into the adult (12^{th}) stage. Upon hatching from the egg, the unsegmented first nauplius stage already possesses three pairs of appendages. At each successive molt these appendages develop by the addition of further setae (hair-like processes on limbs and mouth parts) and/or segments. Rudimentary forms of other appendages and additional body segments (somites) also develop during the nauplius (larval) stages. During the copepodite

(juvenile) stages a new somite is added at each successive molt. The adult copepod bears six pairs of appendages and consists of ten somites (Huys et al. 1996). The time to develop from hatch to adult as well as the generation time (hatch to hatch) varies with temperature, food-availability, population density, *etc.* In general, however, time from hatch to adulthood takes 10-20 days and the generation time is 15-40 days at 15-25 ºC. The shape of the egg, presence and number of egg sacs, the number of eggs within each egg sac as well as the number of broods (from a single fertilization) differs widely between species (Hicks and Coull 1983).

162. Harpacticoid copepods comprise more than 3.000 species, are mostly free-living benthic organisms and are usually the second most abundant group of animals (after nematodes) in marine benthic communities (Huys et al. 1996). They almost always dominate the gut contents of bottom or phytal feeding larval and juvenile fish (Hicks and Coull 1983). Several authors have described toxic effects of single substances, oils, complex effluents etc. on harpacticoid copepods, such as *Amphiascus tenuiremis* (Chandler et al. 2004a, b; Bejarano and Chandler 2003), *Tisbe battagliai* (Hutchinson *et al*., 1999a, b), *Nitocra spinipes* (Bengtsson 1978; Renberg et al. 1980; Tarkpea et al. 1999; Breitholtz and Bengtsson 2001; Breitholtz and Wollenberger 2003), *Nitocra affinis* (Ustach 1979) *and Tigriopus japonicus* (D'Agostino and Finney 1974).

163. Zooplankton communities in the Baltic Sea and other waters are often dominated by calanoid copepods, such as *Eurytemora* spp. and *Acartia* spp. (Voipio, 1981), which provide an important link between phytoplankton and higher communities (e.g. fish) in the pelagic food chain (*e.g.* Størrup *et al.*, 1986). For instance, *Acartia tonsa* has been widely used for ecotoxicological testing owing to its ecological importance and since it is easy to cultivate in the laboratory (Bushong *et al.*, 1990; Kusk and Petersen, 1997). A sensitive larval inhibition test has been developed and used to study effects of *e.g.,* tributyltin (TBT) (Kusk and Petersen, 1997) and estrogens (Andersen *et al.*, 2001). In short, this test covers the period of development from egg until approximately 50 % of the control organisms reach a copepodite stage (after 5-8 days depending on temperature).

5.1.3.1 Protocols using copepod species

164. Acute toxicity tests with copepods have been established as Swedish (SIS 1991) and International Standards (ISO 1997). One chronic test protocol has been established by the American Society for Testing and Materials (ASTM E2317-04; ASTM 2004b).

165. OECD is preparing two test guidelines to standardize procedures for evaluating the effects of chemicals on calanoid (OECD 2004b) and harpacticoid (OECD 2004c) copepods (Annex 3 and Table 4). The calanoid guideline is designed specifically for *Acartia tonsa* and involves exposure of the animals from the egg to adult egg producing stage (F_0 generation). Endpoints for this portion of the test include early life stage development, the onset of egg production and stable egg production, survival, sex ratio, and copepod length. The early life stages of the F_1 generation are exposed and the effects of the test substance on development are evaluated. The primary endpoint associated with this portion of the test is the Larval Development Ratio, which compares the total number of copepodites produced to the total number of early-stage individuals (nauplii + copepodites). The first portion of the test lasts 14–17 days, whereas the second runs 5–7 days. The harpacticoid guideline (Table 6) suggests that *Amphiascus tenuiremis*, *Nitocra spinipes*, and *Tisbe battagliai* are appropriate species to use for this guideline. Newly hatched larvae (<24-h old) are placed individually into microwell chambers (or other suitable containers) and exposed to the test substance. When the larvae mature (about 10–15 days) they are examined to determine gender, and then mating pairs are created and placed into individual microwell chambers. The pairs are exposed to the test substance for 7–14 days, during which time mating occurs. The test continues until the females have produced at least one brood. The numbers of young produced during exposure to the test material are compared to those produced in the control treatments. Additional endpoints that should be recorded are

adult survival and the time to the first brood. Brood size, the proportion of infertile eggs, and time between broods may also be used in the evaluation of a test substance. Basically the same life cycle protocol already exists as an ASTM standard (ASTM 2004b).

5.2 INSECTS AS REPRESENTATIVE AQUATIC INVERTEBRATES

5.2.1 Chironomids as representative insects

166. The family Chironomidae (true or non-biting midges) belongs to the order of Diptera and consists of several thousands of species with a worldwide distribution (Pennak 1989). As insects they represent the species richest and ecologically one of the most important classes of invertebrates. Chironomid larvae (but also other life stages) are an important food source for fish and other predators, which is partly linked to their numerical abundance. The larvae inhabit nearly all freshwaters and often represent more than half of the total number of macroinvertebrate species. The larvae of some species are found in saltwater and a number of species develops in terrestrial habitats as well. Chironomid larvae have a length of 1 to 30 mm (depending on the species and larval stage) and feed on algae, higher plants, and organic detritus (Pennak 1989; Armitage et al. 1995). They undergo a complete metamorphosis (i.e. are holometabolic) and the life stages comprise an egg stage, four larval stages (L1 to L4), pupa and imago. Chironomids are sexually reproducing organisms and the sexes are easily separated since the males have conspicuous plumose antennae. The larvae are sediment dwellers and inhabit different types of sediment depending on physical and nutritional criteria. The four larval stages take up most of the life span, while the imago lives only for a few days (at maximum 15% of the life span). Considering that the short-lived adults have strongly reduced mouthparts, hence only a limited ability to take up food, chironomids may be regarded as true aquatic insects. Adult midges swarm near water bodies and in the swarm mating takes place. Shortly after mating the female produces an egg-rope containing a few hundred eggs, which is usually attached to some substrate at or just below the water surface. After hatching, larvae sink to the sediment or alternately may be carried by the water flow and thus are able to colonize downstream habitats.

5.2.1.1 Protocols using chironimid species

167. Chironomid species such as *Chironomus riparius* and *C. tentans* have been used extensively in the toxicity testing of chemicals. Hence, many data are available for comparison and numerous laboratories and authorities are familiar with this test organism. A great variety of toxicity tests with chironomids have been published. Experimental designs include: a 48-h larval acute test; a 10-d larval survival and growth test; a 28-d emergence and development test up to a 65–d life-cycle study. All test systems include water and sediment, apart from the 48-h test, which is a water-only test. OECD toxicity testing guidelines are available for examining spiked water (OECD 2004e; i.e. OECD TG No 219) and spiked sediment scenarios (OECD 2004d; i.e. OECD TG No 218) (Table 4). For *C. tentans*, standard toxicity testing methods have been published by the US EPA (US EPA 1996b, 1996c). Despite the fact that these tests are chronic, they do not include reproductive parameters. Reproduction is typically covered in life-cycle tests, and effects of different chemicals on reproductive parameters have been published in life-cycle studies with different designs (Taylor et al. 1993; Kahl et al. 1997; Watts et al. 2001; Ristola et al. 2001; Hooper et al. 2003, 2005; Hruska and Dube, 2005). The US EPA (2000) published a method for conducting a life-cycle test with *C. tentans* (background information provided in Benoit et al., 1997; Sibley et al. 1997, 2001) and Taenzler et al. (2006) and Weltje et al. (2006) developed a life-cycle testing method for *C. riparius*, based upon the OECD TG 219 (Table 5). A step further is the multi-generation test, which was conducted by Postma and Davids (1995) who studied adaptation to cadmium in nine consecutive *C. riparius* generations. Finally, it is possible to use deformities in the mouthparts of the larval head capsule as a sublethal parameter for toxicant exposure. These deformities are mainly linked to the presence of metals in sediments, as was demonstrated for field-collected larvae, but it is also observed in laboratory

assays. Interestingly, the vertebrate estrogen 4-*n*-nonylphenol induced mentum deformities in a concentration-dependent manner (Meregalli et al. 2001), while 17α-ethynylestradiol did not (Meregalli and Ollevier 2001).

168. The endocrine system of insects is the best described within the invertebrates. This is related to the silk-worm industry and the development of 3rd generation insecticides that specifically target the insect endocrine system. These insecticides act as juvenile hormone (ant)agonists or ecdysone (ant)agonists, and interfere with various processes, including molting, metamorphosis, vitellogenesis and reproduction. Third generation insecticides can be used as reference chemicals for inducing effects on endocrine processes in insects. In addition, a co-exposure of the test compound with an antagonistic reference chemical can help elucidating a working mechanism. The available knowledge on the insect endocrine system and the availability of specific reference chemicals is a major advantage of working with aquatic insects on endocrine disruption in invertebrates. Furthermore, the size of chironomid larvae allows for making biochemical measurements, such as vitellogenesis, to generate evidence on the endocrine working mechanism of the chemical under investigation (Hahn and Schulz 2002; Hahn et al. 2002). Moreover, a cell line of *C. tentans* is available to test the interactions of chemicals with the ecdysteroid receptor (Quack et al. 1995; Spindler-Barth et al. 1998). This cell line could be used to identify potential endocrine disruptors for arthropods. Finally, population growth models are available (e.g. Pery et al. 2002, 2005; Ducrot et al. 2004; Forbes and Cold 2005) that can be used to analyze the data from life cycle tests and assess the importance of the observed effects on a population level. In the end this is more important than clarifying the exact working mechanism.

5.2.2 Protocols using other sediment-dwelling species

169. The amphipod *Hyalella azteca* has been used in chronic toxicity testing to study potential sublethal effects of contaminated sediments. As e.g. C. tentans this species is an ecologically important sediment dweller in freshwater systems. The protocol includes survival, growth, and reproductive endpoints (Table 5). The species has been used successfully in EDC testing, although not necessarily following procedures similar to this protocol. The EDC testing already performed on this species highlights the utility of including sediment-dwelling species in EDC evaluations. Similar protocols have been developed by Environment Canada (1997a, 1997b).

170. *Leptocheirus plumulosus*: US EPA (2001) developed a chronic protocol to evaluate the potential sublethal toxicity of contaminated sediments to an estuarine amphipod, *Leptocheirus plumulosus* (Table 5). The procedure runs for 28 days and includes survival, growth rate, and reproductive endpoints. The test requires sediment as a substrate for the amphipods, but can be used with sediments having a wide variety of pore water salinities (1‰–35‰). This protocol could be adapted to the EDC program to test the potential effects of sediment-bound EDCs (created by spiking sediments) on a sediment-dwelling estuarine organism (Table 5).

Table 3. Recommended Mysid Life Cycle Toxicity Test Conditions

	ASTM E1191 (ASTM 1997)	OPPTS 850.1350 (US EPA 1996)
Test Species:	*Americamysis bahia* *A. bigelowi* *A. almyra*	*Americamysis bahia*
Holding Conditions:	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 3ºC/12 h salinity at <3‰/24 h) 76-L aquaria Flow through or recirculating system 14 h light:10 h dark, or 16 h light: 8 h dark, with 15–30 min transition period Gentle aeration Feed excess ≤24 h old *Artemia;* 150/mysid/day; may supplement with algae or other food	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 1ºC/24 h; salinity at <5‰/24 h) Flow through or recirculating system 14 h light:10 h dark, with 15–30 min transition period Aeration if needed
Test Setup:		
Test organism age:	≤24 h	≤24 h
Duration:	≥7 days after median first brood release in controls	28 days
Test Material:	Reagent grade or better	NS [a]
Endpoint(s):	Survival, growth, reproduction	Survival, growth, young produced
Number of Treatments:	≥5 plus control (add solvent control if necessary)	≥5 plus control (add solvent control if necessary)
Concentration Series:	Test concentrations should bracket the highest concentration at which there is not an unacceptable effect; each concentration should be at least 50% of the next highest concentration	5 or more concentrations chosen in geometric ratio between 1.5 and 2.0.
Dilution Water: Solvent:	Natural or reconstituted seawater acceptable to saltwater mysids; uniform quality during test; should not affect test outcome Must allow satisfactory survival, growth, and reproduction If solvent used, ≤0.1 mL/L concentration.	Natural (>20-µm-filtered) or artificial seawater If solvent used, ≤0.1 mL/L concentration
Flow Conditions:	Flow through	Flow through
Delivery System: Flow Rate: Calibration limit: Calibration/ Check:	Proportional diluter >5 volume additions/24 h (must be capable of 10 additions/24 h) <10%/chamber/time Prior to test; visual check twice daily	Proportional diluter 5 × chamber volume/24 h <10%/chamber/time Prior to test; twice daily
Number of Replicates:	Variable, estimated according to expected variation, desired detection limit, and selected power.	5+ (minimum 40 mysids/treatment)
Test Chamber:	e.g., 300 mm × 450 mm × 150 mm deep with adequate compartments (to provide 30 cm^2/mysid).	Volume NS; materials must minimize sorption of test chemicals; loosely covered
Test Volume:	Solution depth ≥100 mm (in above specified chamber)	NS
Number of organisms/rep:	NS (recommends 1 male-female pair/ compartment, but can't determine gender for~12 d)	8 (maximum)
Other Setup Notes:	NS	NS
Initiation Notes:	NS	NS
Test Conditions:		
Light:	NS	NS
Photoperiod:	14 h light:10 h dark, or 16 h light:8 h dark, with 15–30 min transition period	14 h light:10 h dark with 15–30 min transition period

	ASTM E1191 (ASTM 1997)	OPPTS 850.1350 (US EPA 1996)
Temperature:	27ºC (for *A. bahia*); 3ºC individual measurements: ±1ºC time-weighted average; <2ºC difference between any two jars measured concurrently.	25°C ± 2ºC
pH:	6.6–8.2	NS
Dissolved Oxygen: Aeration:	A concentration between 60–100% of saturation is best. yes	60–105% saturation
Salinity:	15–30‰; variation among treatments should be <5, must be <10 ‰	20‰ ± 3‰
Monitoring:		
Test Concentration	Twice prior to test, at 24 h apart; Measured concentration ≤30% of nominal concentration. During test frequently enough to establish average and variability, at least weekly.	At Day 0, 7, 14, 21, 28; should vary <20% among replicates/concentration
WQ Frequency:	Salinity daily; temperature in one chamber hourly or min/max measured daily; pH at start and end of test and weekly in control, include highest concentration; dissolved oxygen in at least one test chamber at start and end and weekly	Weekly (includes pH)
Observation Frequency:	Daily Count, determine gender, remove dead G1 mysids; count live mysids; record number live females Record day of brood release; count and remove young daily Record abnormal development and aberrant behavior	Periodically; record number dead on Day 7, 14, 21, 28
Feeding:	Live brine shrimp nauplii at least once daily; may supplement Dead brine shrimp should be removed daily before feeding occurs.	Recommend 48-h-old *Artemia.* Frequency and amount not specified.
Other Monitoring Notes:	Weekly determinations of particulate matter, total organic carbon, and total dissolved gasses desirable	NS
Termination Notes:	Count live G1 mysids and determine gender Desirable to measure total body length (anterior tip of carapace to tip of uropod) Obtain dry weight of surviving G1 (males and females separate); remove any brine shrimp present; rinse mysids in deionized water, dry at 60ºC for 72–96 h Morphological observations at end of test may be desirable May be desirable to hold G2 mysids for 4+ day longer to observe possible effects	Record number of dead on Day 7, 14, 21, 28 Record number of males & females and measure body length (anterior tip of carapace to tip of uropod) when distinguishable and on Day 28. Count and separate G1 offspring as produced, hold at test concentrations. If possible (i.e., by Day 28), count, determine gender and measure G2 mysids. Record abnormal behavior or morphology.
Test Validity Criteria:	A test is valid if General test requirements are met ≥70% G1 control survival ≥75% G1 control females produce young ≥3 average number of young/female	A test is valid if ≥75% G1 control females produce young ≥3 average number of young/female/day
Range-Finding Test		
Concentration Series number of samples test volume test containers number of animals/rep duration	NS	Widely spaced; e.g., 1, 10, 100mg/L 1 NS NS Minimum 10/concentration NS, allow estimate of test concentrations

	ASTM E1191 (ASTM 1997)	OPPTS 850.1350 (US EPA 1996)
Termination Notes:	NS	NS
Test Validity Criteria:	NS	NS
Reference Toxicant:	NS	NS
Concentration Series number of reps test volume test containers number of animals/rep	NS	NS
Termination Notes:	NS	NS
Test Validity Criteria:	NS	NS

a) NS Not specified.

Table 4. Example OECD protocoles for evaluating chronic toxicity in aquatic arthropods.

	OECD TG 211 September 1998	**OECD TG218/219 February 2001**	**OECD Draft Calanoid TG**	**OECD Draft Harpacticoid TG**
Test Species:	*Daphnia magna*	*Chironomus riparius*, *C. tentans*, *C. yoshimatsui*	*Acartia tonsa*	*Amphiascus tenuiremis, Nitocra spinipes, Tisbe battagliai*
Holding/ Culturing Conditions:	Hold at conditions similar to test	Hold at conditions similar to test	Hold at conditions similar to test	Hold at conditions similar to test
Test Setup:				
Test organism age:	<24 h; must not be first brood progeny	1st instar larvae	1st stage nauplii	Newly hatched nauplii (<24-h old)
Duration:	21 d	20–28 d; 28–65 d depending on species. If midges emerge earlier, test can be terminated five days after emergence of the last adult in the control.	19–25 d	20–25 d
Test Material:	Chemical in water	Spiked Sediment (218) or spiked water (219)	Seawater (natural or artificial)	Seawater (natural or artificial)
Endpoint(s):	Primary: Number of offspring per adult alive at end of test Other: parent survival; time to first brood Draft enhanced endpoints for endocrine disruption evaluations: offspring sex ratio; molting inhibition	Total adult emergence; development time	F0: (14–17 d) early life-stage development, onset of egg production and stable egg production, survival, sex ratio, body length F1: (5–8 d) development (larval development ratio, LDR)	Reproductive output of exposed animals versus control(s) Parent animal survival Time to first brood should also be reported. Also, brood size, infertile or unhatched eggs, time interval between successive broods and possibly intrinsic or instantaneous rates of population increase (rm or λ), may be examined
Number of Treatments:	≥5 and a laboratory control	≥5 and a laboratory control	≥5 and a laboratory control	≥5 and a laboratory control

	OECD TG 211 September 1998	**OECD TG218/219 February 2001**	**OECD Draft Calanoid TG**	**OECD Draft Harpacticoid TG**
Concentration Series:	Geometric series with separation factor ≤3.2; based on acute test or range-finding test	Factor between concentrations should be ≤2 for EC_x studies; ≤3 for LOEC/NOEC studies	Geometric series with separation factor ≤3.3 Test concentrations should not include any that have a statistically significant effect on survival of the F_0 generation since the main objective is to measure sublethal effects	Geometric series with separation factor ≤3.2 Test concentrations should not include any that have a statistically significant effect on survival since the main objective is to measure sublethal effects
Dilution Water: Solvents: Dispersants:	May be used to make stock for dosing test (e.g., acetone, ethanol, methanol) May assist in accurate dosing and dispersion (e.g., Cremophor RH40, HCO-40)	Any suitable natural or synthetic water may be used. The water has to be aerated before use. May be used to make stock for dosing test (e.g., acetone, ethanol, methanol)	DO saturation value >70%; pH = 8.0 ± 0.3 before use	Constant quality; DO saturation value >70%; pH = 8.0 ± 0.3 before use
Flow Conditions:	Semi-static; flow-through allowable	Static	Static-renewal	Static-renewal
Number of Replicates:	10 (semi-static); 4 (flow through)	≥3 for EC_x study; ≥4 for LOEC/NOEC study	Control: minimum 6, 12 recommended for LDR test Test substance: depends on design; 4 (linear regression), 6 (ANOVA)	Minimum 3 microplates/ concentration (48–144 wells)
Test Chamber:	Glass beakers; volume not specified	600-mL, 8-cm diameter glass beaker	100-mL beaker	Microwell (300–5000 µL total volume) test chambers preferred
Test Volume:	50–100mL	1.5–3.0 cm sediment depth	80 mL	<50 mL
Number of organisms/rep:	1 (semi-static); 10 (flow through)	20 for *C. riparius* and *C. yoshimatsui*; 12 for *C. tentans*	10	48 animals added to three replicate test vessels (microplates) per concentration and control(s)
Other Setup Notes:	Renew test solution at least 3 times/week; more frequently if not stabile (<80% initial concentration over 3 d) Prepare new test vessels and transfer adults to them	Formulated sediment (also called reconstituted, artificial or synthetic sediment) should be used. Spike sediment for TG218; spike water for TG219	LDR test: renew 50–80% on Day 3 or increase volume Basic exposure: renew 50–80% every 2nd day or increase volume Prepare new test vessels and transfer adults to them	Depends on stability of test substance, should be at least three times per week Prepare new test vessels and transfer adults to them With microplates, evaporation losses ≤ 7% daily; correct by adding deionized or distilled water

	OECD TG 211 September 1998	OECD TG218/219 February 2001	OECD Draft Calanoid TG	OECD Draft Harpacticoid TG
Test Conditions:				
Light:	Ambient laboratory illumination <15-20 µE/m^2/s	About 500 to 1000 lux	Wide-spectrum fluorescent lights; low intensity; ~5-10 µmol/s/m^2	Not specified
Photoperiod:	16L:8D	16L:8D	12L:12D	*N. spinipes*–0L:24D *A. tenuiremis*–12L:12D *T. battagliai*–16L:8D
Temperature:	18–22°C; should not vary >2°C during any one test	20°C ± 2°C; 23°C ± 2°C for *C. tentans*	15–20°C; vary < 2°C during test	20-25ºC; vary ≤ 2°C during test
pH:	6–9; should not vary >1.5 units	6–9 at start of test	8.0 ± 0.3	± 0.3 units of control
Dissolved Oxygen: Aeration:	3 mg/L None allowed	>60% saturation value Gentle aeration; 1 bubble/second	>70% saturation value if DO level falls below 70% saturation: 1 bubble/second	>70% saturation value (dilution water) None during test
Hardness/ Salinity:	>140 mg/L $CaCO_3$	<200 mg/L $CaCO_3$	~20 ‰ ± 2 ‰	Use same as culture water; *N. spinipes* 1–35‰ *A. tenuiremis* 15–35‰ *T. battagliai* 20–35‰
Monitoring:				
WQ Frequency:	DO, temperature, hardness, pH: once per week in fresh and old media, controls, and highest test concentration Determine concentration of test substance regularly	DO measured daily in all test vessels Hardness and ammonia at the beginning and at the end of the test in controls and one vessel of highest concentration. Temperature and pH at start and end of test.	Measure dissolved oxygen, pH, salinity, and temperature in controls and all test concentrations each time test medium is renewed. As a minimum, measurements shall be made in the control(s) and highest test concentration. Temperature preferably monitored continuously.	Measure dissolved oxygen, pH, and temperature should be measured in the controls and all test concentrations each time test medium is renewed. As a minimum, measurements should be made in the control(s) and highest test concentration. Temperature preferably monitored continuously in at least one test vessel
Observation Frequency:	Not specified	At least three times per week; visual assessment of abnormal behaviour (e.g. leaving sediment, unusual swimming), compared with the control.	Daily for egg production test; not specified for LDR test	Not specified

	OECD TG 211 **September 1998**	**OECD TG218/219** **February 2001**	**OECD Draft Calanoid TG**	**OECD Draft Harpacticoid TG**
Feeding:	At least 3 times per week, preferably daily (semi-static); concentrated algal suspension	At least 3 times per week, preferably daily; 0.25-0.5 mg fish-food (a suspension in water or finely ground food) per larvae per day for the first 10 days. Slightly more food for older larvae; 0.5–1.0 mg per larvae per day Reduce food ration in all treatments and control if fungal growth occurs or if mortality is observed in controls. If fungal development cannot be stopped the test is to be repeated.	LDR test: 5 × 104 cells/mL of medium added on days 0 and 3 Basic exposure: 5 × 104 cells/mL of medium added on day 0 and subsequently to every renewal of the medium. Egg production: 5 × 104 cells/mL of medium added daily.	A. tenuiremis: 2 µL of 1:1:1 volume (107 cells/mL) chlorophyte, chrysophyte, diatom suspension every 6 d during maturation/development and reproduction. N. spinipes: (~107 cells/mL) chrysophyte, preferably Rhodomonas salina or R. baltica, every third day during maturation/development and reproduction; final concentration in each test chamber is 105 cells/mL. Tisbe battagliai: (~3 × 107 cells/mL) chrysophyte, preferably Rhodomonas reticulata, every third day during maturation/development and reproduction, so that the final concentration in each test chamber is 2 ×105 cells/mL.
Other Monitoring Notes:	Record parent animal mortality daily Remove and count offspring daily Record presence of aborted eggs or dead offspring daily	During the period of expected emergence, a daily count of emerged midges is necessary.		
Termination Notes:		Record number of fully emerged male and female midges	Fix animals in Lugol's solution; score biological parameters (count, measure, determine gender, etc.)	Fix animals in Lugol's solution or formaldehyde (4%); score biological parameters (count, measure, determine gender, etc.)

	OECD TG 211 September 1998	OECD TG218/219 February 2001	OECD Draft Calanoid TG	OECD Draft Harpacticoid TG
Test Validity Criteria:	Parent animals (female Daphnia) mortality ≤ 20% at the end of the test Mean number of live offspring produced per parent animal surviving at the end of the test > 60	Control mortality ≤30% at end of test C. riparius and C. yoshimatsui emergence to adults occurs 12–23 days after initiation; C. tentans emergence to adults occurs 20–65 days after initiation Mean emergence in controls 50−70 per cent DO concentration ≥60% air saturation value at the end of the test pH of overlying water 6–9 range in all test vessels Water temperature vary ≤ 1.0 °C between vessels during test Temperature held within the ranges specified for test species	Mortality in mass culture control(s) ≤20% at end of exposure DO concentration ≥70% air saturation value throughout the exposure period Temperature vary ≤ 2°C during the test pH vary ≤ 0.3 units from average control pH Salinity vary ≤ 10% from the control start value Sex ratio in controls = 40:60 to 60:40) Hatching success in hatching control ≥ 80% Average mortality in the control(s) on day of LDR observation ≤ 20% Average number of eggs/female /d in controls ≤ 30 at Day 12 and later Control mortality of the isolated females in the egg production test (Day 7–12) ≤ 20%.	Control mortality ≤ 20% at the end of the exposure period DO concentration ≥70% air saturation value throughout the exposure period Temperature vary ≤ 2°C during the test pH vary ≤ 0.3 units from average control pH Salinity vary ≤ 10% from the control start value Sex ratio in controls = 40:60 to 60:40) Average number of offspring per clutch in the controls ≥ 5 Average number of days to first brood in the control(s) ≤ number of days = 50% of total exposure period (at a temperature of 20°C)

Table 5. Example US EPA protocols for evaluating chronic toxicity in Aquatic Arthropods

	US EPA-821-R-02-013 October 2002	US EPA/600/R-99/064 March 2000	US EPA/600/R-99/064 March 2000	US EPA 600/R-01/020 March 2001
Test Species:	*Ceriodaphnia dubia*	*Chironomus tentans*	*Hyalella azteca*	*Leptocheirus plumulosus*
Holding Conditions:	Hold at conditions similar to test	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 1 ºC/1-2 h)	Hold at conditions similar to test or acclimate gradually to test conditions (Temperature at 1 ºC/1-2 h)	Hold at conditions similar to test or acclimate gradually to test conditions (A change in temperature or salinity not exceeding 3 °C or 3 ‰ per 24 h)
Test Setup:				
Test organism age:	<24 h and all within 8 h of the same age	1 d (<24 h)	7 to 8 d	Neonates: age-selected (<48 h old) or size-selected: retained between 0.25 mm and 0.6 mm mesh screens
Duration:	Maximum of 8 d Until 60% or more of surviving control females have three broods	50–65 d depending on emergence. Each treatment may need to be terminated separately	42 d	28 d
Test Material:	Effluents and receiving waters	Sediment	Sediment	Sediment
Endpoint(s):	Survival and reproduction	20-d survival and weight; female and male emergence, adult mortality, the number of egg cases laid, the number of eggs produced, and the number of hatched eggs	28-d survival and growth; 35-d survival and reproduction; and 42-d survival , growth, reproduction, and number of adult males and females on Day 42	Survival, growth, and reproduction
Number of Treatments:	Effluents: 5 and a control Receiving Water: 100% receiving water (or a minimum of 5) and a control			
Concentration Series:	Effluents: ≥ 0.5 Receiving Waters: None or ≥ 0.5			
Dilution Water: Solvent:	Uncontaminated source of receiving or other natural water, synthetic water prepared using Millipore milli-Q® or equivalent deionized water and reagent grade chemicals or DMW			

	US EPA-821-R-02-013 October 2002	US EPA/600/R-99/064 March 2000	US EPA/600/R-99/064 March 2000	US EPA 600/R-01/020 March 2001
Flow Conditions:	N/A	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)	2 volume additions/d; continuous or intermittent (e.g., one volume addition every 12 h)	Siphon off and replace 400 mL 3 times/week
Number of Replicates:	10 (required minimum)	16 (12 at Day -1 and 4 for auxiliary males on Day 10)	12 (4 for 28-d survival and growth and 8 for 35- and 42-d survival, growth, and reproduction)	5 for toxicity test; ≥2 dummy chambers for pore water ammonia (Day 0 and Day 28)
Test Chamber:	30-mL borosilicate glass beakers or disposable polystyrene cups (recommended because they fit in the viewing field of most stereoscopes).	300-mL high-form lipless beaker	300-mL high-form lipless beaker	1-L glass beaker or jar with 10cm inner diameter
Test Volume:	15 mL (minimum)	100 mL sediment, 175 overlying water	100 mL sediment, 175 mL overlying water in the sediment exposure from Day 0 to Day 28 (175 to 275 mL in the water-only exposure from Day 28 to Day 42)	175 mL (about 2cm depth) sediment, approximately 725 mL water (fill to the 900mL mark on jar)
Number of organisms/rep:	1 Assigned using blocking by known parentage	12	10	20
Other Setup Notes:	New test solutions are prepared daily, and the test organisms are transferred to the freshly prepared solutions			
Test Conditions:				
Light:	Ambient laboratory illumination 10–20 µE/m^2/s, or 50–100 ft-c	Wide-spectrum fluorescent lights; About 100 to 1000 lux	Wide-spectrum fluorescent lights; About 100 to 1000 lux	Wide-spectrum fluorescent lights; About 500 to 1000 lux
Photoperiod:	16L: 8D	16L: 8D	16L: 8D	16L: 8D
Temperature:	25 °C±1 °C	23 °C ± 1 °C	23 °C ± 1 °C	Daily limits: 25 °C (±3 °C); 28-d mean: 25 °C (±2 °C).
pH:				7.0 to 9.0
Dissolved Oxygen: Aeration:	4.0 mg/L used only as a last resort	>2.5 mg/L if DO level falls below 2.5 mg/L: 1 bubble/second	>2.5 mg/L if DO level falls below 2.5 mg/L: 1 bubble/second	Daily Limits: ≥3.6 mg/L (50% saturation) 28-d mean: ≥4.4 mg/L (60% saturation)

	US EPA-821-R-02-013 October 2002	US EPA/600/R-99/064 March 2000	US EPA/600/R-99/064 March 2000	US EPA 600/R-01/020 March 2001
Salinity:	N/A	N/A	N/A	Daily limits: 5‰ (± 3‰) if pore water is 1‰ to 10‰, 20‰ (±3‰) if pore water is 10‰ to 35‰; 28-d mean: 5‰ (± 2‰) or 20‰ (± 2‰)
Monitoring:				
WQ Frequency:	DO, temperature, pH: daily, prior to renewals in at least one test chamber at each concentration and control pH is measured in the effluent sample each day before new test solutions are made Conductivity, alkalinity, and hardness measured in each new sample and in the control	Hardness, alkalinity, conductivity, and ammonia at the beginning, on Day 20 and at the end of the test. Temperature daily (ideally continuously). DO and pH three times/week. Conductivity weekly. Measure DO more often if DO has declined by more than 1 mg/L since previous measurement.	Hardness, alkalinity, conductivity, and ammonia at the beginning and end of a sediment exposure (day 0 and 28). Temperature daily. Conductivity weekly. DO and pH three times/week. Measure DO more often if DO has declined by more than 1 mg/L since the previous measurement.	Daily temperature in water bath or test or surrogate chamber, daily min/max recommended; salinity, temperature, DO, and pH at test initiation and termination, and in one replicate per sediment treatment preceding water renewal during the test (three times per week); aeration rate daily in all containers; total ammonia on Days 0 and 28 in one replicate per treatment.
Observation Frequency:	Daily	Daily to assess test organism behavior such as sediment avoidance	Daily to assess test organism behavior such as sediment avoidance	3 times/week in each test chamber preceding water renewal for condition and activity
Feeding:	0.1 mL each of YCT and algal suspension per test chamber daily	1.5 mL of Tetrafin® (4 mg/mL dry solids) to each beaker. If fungal or bacterial growth develop from excess food, feeding should be suspended for one or more days. If feeding is suspended in one treatment, it should be suspended in all treatments.	YCT food, fed 1.0 mL (1800 mg/L stock) daily to each test chamber. If fungal or bacterial growth develop from excess food, feeding should be suspended for one or more days. If feeding is suspended in one treatment, it should be suspended in all treatments.	3 times/week after water renewal Days 0–13, 20mg TetraMin® per test chamber; Days 14–28, 40mg TetraMin® per test chamber.
Other Monitoring Notes:		At 20 d, 4 of the initial 12 reps. are selected for use in growth and survival measurements. AFDW of midges should be determined for the growth endpoint. Emergence traps are placed on the reproductive replicates on day 20		

	US EPA-821-R-02-013 October 2002	**US EPA/600/R-99/064 March 2000**	**US EPA/600/R-99/064 March 2000**	**US EPA 600/R-01/020 March 2001**
Termination Notes:	Because of the rapid rate of development of Ceriodaphnia dubia, at test termination all observations on organism survival and numbers of offspring should be completed within two hours.	Clean sediment will typically require 40–50 d from initial setup to completion (emergence). Environmental stressors will reduce growth and delay emergence. For treatments in which emergence has occurred, the treatment (not the entire test) is ended when no further emergence is recorded over a period of 7 d.	Growth can be reported as either length or weight Length should be measured ± 0.1 mm from the base of the first antenna to the tip of the third uropod along the curve of the dorsal surface. Dry weight of amphipods in each replicate can be determined on Day 28 and 42.	Missing adult organisms should be recorded as dead Growth rate of amphipods can be reported as daily change of average individual length or weight. Growth Rate (mg/individual/day) = (mean adult dry weight - mean neonate dry weight)/28 Count offspring within 2 weeks of termination.
Test Validity Criteria:	Mean control survival ≥ 80%; 60% of surviving control females must produce at least three broods, with an average of 15 or more young per surviving female.	Average size of C. tentans in the control at 20 d ≥ 0.6 mg/surviving organism dry weight or 0.48 mg/ surviving organism AFDW. Emergence ≥ 50%; mean number of eggs/ egg case ≥ 800; percent hatch ≥ 80% Hardness, alkalinity, and ammonia in overlying water should not vary by more than 50% during the test, DO > 2.5 mg/L	Mean control survival ≥ 80% on Day 28. Hardness, alkalinity, and ammonia in the overlying water typically should not vary by more than 50% during the sediment exposure, and dissolved oxygen should be maintained above 2.5 mg/L in the overlying water.	Mean control survival ≥ 80% at the end of the test, with no single replicate having 60% survival or less.

6.0 DESCRIPTION OF ASSAY ENDPOINTS REFLECTIVE OF REPRODUCTIVE AND DEVELOPMENTAL IMPAIRMENT

171. In considering the list of potential endpoints presented in the following sections, it is desirable that the measured response is directly related to exposure to the potential EDC and that the response is the result of interruption of endocrine function (Ingersoll et al. 1999). Although many, if not all of the endpoints described in the following sections could indicate a response to an EDC, most also will vary in response to exposure to other stressors. Further confounding the interpretation of testing results is the interrelatedness of some endpoint measurements. Thus, a stressor could act on a test organism to reduce its swimming ability, thereby reducing its ability to feed, which then reduces its growth. Similarly, reduced fecundity could be an expression of reduced growth. Often, exposure to a compound that results in reduced fecundity also results in reduced growth (Nimmo et al. 1981); however, this is not always the case. Lussier et al. (1999) reported fecundity effects without reduced growth, and McKenney and Celestial (1996) found reduced reproductive measures at concentrations of methoprene below that which resulted in reduced growth. Various endpoint measures can have differing sensitivities to stressors. For example, reproductive parameters are often, but not always more sensitive measures of contaminant toxicity than is simple survival (Lussier et al. 1985). Hollister et al. (1980) found no reproductive impairment among test organisms, in spite of significant mortality due to exposure to alkaline effluent. Additionally, stressors can affect some reproductive measures, whereas others cannot (e.g., Hollister et al. 1980, Lussier et al. 1985). Clearly, the possible interrelationships among potential endpoints and the varying sensitivities shown by some endpoints underscore the need to use multiple endpoints in EDC testing. Examples of assay endpoints reflective of reproductive and developmental impairment are given below. The list is not complete but rather involves general endpoints identified in the protocols described in section 5.

6.1 GROWTH, MORPHOLOGICAL AND BEHAVIORAL ALTERATIONS

172. Measures of growth, morphological changes, and changes in test organism behavior as indicators of EDC effects are reviewed below.

6.1.1 Growth

173. Molting is one of the key arthropod physiological processes that are under hormonal control, and therefore, it is susceptible to the negative effects of EDCs. Molting is regulated primarily by the interaction of molt stimulating hormones (ecdysteroids) and nervous system secretions produced in the cephalothorax with molt inhibiting hormones produced in the eyestalks (e.g., Cuzin-Roudy and Saleuddin 1989, Fingerman 1997, Subramoniam 2000, Zou and Fingerman 1997). Molting is either directly or indirectly involved in the expression of the various endpoints that may be examined through toxicological testing. Because noticeable growth can only occur as a result of molting, any disruption of molting could result in alterations in growth. Many pesticides, generally classed as Insect Growth Regulators (IGR), have been developed that target insect development. IGRs function as ecdysone agonists, JH or anti-JH analogs and as chitin synthesis inhibitors. All can have detrimental effects on crustaceans, especially through interruption of the molt cycle, most likely by impacting endocrine function (Touart 1982). For this reason, the estimation of molting frequency may be a useful endpoint relevant to mysid and other crustaceans for EDC testing. The duration of the molt cycle of adult mysids (*A. bahia*) was determined to be about 6.6 d (Touart 1982), with the duration for male being slightly less and that for females being slightly more. Juveniles are expected to molt within the first 24 h post release, but may delay molting until about 48 h (Touart 1982). Successive juvenile molts occur at increasingly longer durations. Touart (1982) found that at test conditions of 22ºC and 20‰, sexual characters were noticeable after the fourth molt (9–12 d) and that mating occurred after the fifth or sixth molts (17–19 d). Higher test temperatures would

likely shorten the duration between molts. Molt staging relies on changes in the integument and is most commonly divided into four stages: postmolt, intermolt, premolt, and molt (Verslycke et al. 2004a). Winkler and Greve (2002) found that temperature, body size, and age all are factors contributing to mysid growth rates. Touart also found that the pesticide diflubenzuron (Dimilin®) increased the duration of the molt cycle in *A. bahia*, probably acting on the mysid endocrine system as a molt inhibitor. This was demonstrated by his experiment in which eyestalk-ablated mysids that were then exposed to dimilin had molt cycle durations that did not differ from those in control animals. In effect, the Dimilin may have replaced or mimicked the "natural" mysid molt inhibitor synthesized in the eyestalks. Zou and Fingerman (1997) suggest a certain degree of overlap between molting hormones and estrogen-mimicking xenobiotics. They tested this hypothesis using two compounds, DES and endosulfan. Molting time for daphnids exposed to DES and endosulfan was delayed because these compounds blocked the ecdysteroid receptors, preventing molting hormones from binding to the receptor and turning the receptor on, thereby slowing the molting process. However, it is unclear whether the delayed molting process was due to the exposure to these contaminants or just a general response to stressors. More pharmacokinetic studies are underway to investigate delayed molting further.

174. Because there are current immunoassay methods for quantifying ecdysteroids and also methods for molt staging, evaluations of potential interactions of EDCs and molting should be possible (Block et al. 2003; Verslycke et al. 2004a).

175. Two direct measures of growth may be used in the assessment of sublethal effects on crustaceans. Probably the most common measurement is the determination of simple dry weight. At test termination, animals are briefly rinsed in deionized water to remove salt, and dried to constant weight. In published studies that employ this method, drying temperatures ranged from 60ºC (e.g., McKenney 1982) to 105ºC (Khan et al. 1992) and drying times ranged from 16 h (Khan et al. 1992) to 72 h (UCSC 1998). ASTM (1997) specified that growth should be measured by drying animals at 60ºC for 72 h to 96 h, or to constant weight. ASTM also recommended that separate determinations be made for males and females. Ash-free dry weight (AFDW) is often used as the appropriate weight measurement for many invertebrates, because the technique reduces any inaccuracies introduced by inorganic constituents in the organism's body. Inorganic components can originate from processes such as the development of skeletal components or feeding (the ingestion of sediment). As with some other small crustaceans, the small size of the mysids and their planktonic diet may make the removal of ash from the dry weight measurement an unnecessary step that would not greatly improve the accuracy of the measurement (US EPA 2000).

176. Another direct measurement of growth is body length. Body length has been measured as the distance from the base of the eyestalks to the tip of the telson (Hunt et al. 1997; UCSC 1998) or to the tip of the exopod (Langdon et al. 1996; Winkler and Greve 2002). ASTM (1997) suggests that body length should be measured along the midline of the body from the tip of the carapace to the tip of the exopod (excluding the terminal setae). ASTM notes that it is difficult to make this measurement on preserved animals because of the body curvature that results from the fixation process. Langdon et al. (1996) reduced the potential inaccuracy in this measurement by relaxing the mysids prior to fixation and then determining length as the sum of a series of relatively straight-line measurements. Dahl et al. (in press) defined the total body length (µm) of the harpacticoid copepod *N. spinipes* exposed to sublethal concentrations of the drug Simvastatin as the distance between the anterior part of prosome to the posterior part of the last urosomite. The study showed that the body length was a less sensitive endpoint than development rate, growth rate and RNA content. Winkler and Greve (2002) anaesthetized mysids in soda water to relax organisms prior to collecting length measurements. Age-specific growth rates were calculated using duration of the successive intermolt periods in days and the growth factor at each molt. Comparisons of different temperatures (10°C and 15°C) different size classes, and other mysids provided a combination of results. The mysid *N. integer* reached maturity in a shorter time and at a smaller size than *P. flexuosus*. *P.*

flexuosus grew faster at all stages but matured much later. Both species had temperature-dependent growth curves but in opposite ways. *N. integer* had a lower growth rate at 10°C than at 15°C.

177. The most common response to exposure to toxicants is reduced growth (several studies) although it may not be a particularly sensitive endpoint (Lussier et al. 1999). McKenney and Celestial (1996) reported reduced growth after exposure to methoprene, a juvenile hormone analog. Reduced growth has important connections to reproductive success because the size of females is directly related to fecundity (Verslycke et al. 2004a). However, reduced growth occurred only at the highest concentration in their exposure series. It may not be useful to measure growth for all treatments. Because growth is a sublethal endpoint, its use has sometimes been restricted to treatments for which survival was not significantly less than that in control treatments (Hunt et al. 1997). Hunt et al. (1997) observed that individuals that survive high toxicant concentrations may often be larger than average. In a study conducted by Hunt et al. (2002), survival of the mysid, *Mysidopsis intii*, was the most sensitive of five endpoints measured during a 28-day chronic exposure to nickel. Growth is less sensitive in such cases because surviving mysids are large, more tolerant of toxicants, and use cannibalism as a nutritional source. The effect of contaminant exposure on growth may be related to test organism age in addition to contaminant concentration. McKenney (1986) found that older (9–16 d) juvenile mysids showed significantly reduced growth at a lower fenthion concentration than that found for younger (0–9 d) juveniles. Reduced growth in mysids possibly results from a transfer of energy from growth mechanisms as an organism attempts to counteract stress (McKenney 1985, 1989, McKenney and Matthews 1990; Section 6.4.3 below). Reduced growth has strong implications for reproductive success in mysids as several studies (e.g., Mees et al. 1994, Turpen et al. 1994) have shown that fecundity is directly related to female body size.

178. Molt stage has become an increasingly used technique for measuring growth. If molt stages are classified, duration of different stages under normal laboratory conditions, and then environmental effects on relative duration of stages can be evaluated, using the molt-stage technique (Gorokhova 2002). Molt cycle chronology is a prerequisite for the use of molt staging in growth studies. One study was conducted using laboratory reared juveniles and subadults of *Mysis mixta* and *Neomysis integer;* molt staging techniques were used to determine the main molt stages for each species under different regimes of temperature and feeding. Field application of the molt stage technique was applied for *Mysis mixta*, to determine molt cycle duration.

6.1.2 Morphology

179. Changes in morphology resulting from exposure to contaminants have been documented for many taxa, including arthropods. For example, chironomid (midge) larvae, which are commonly used in toxicological testing, can develop mouthpart deformities when exposed to chemicals that affect endocrine systems (Meregalli et al. 2001, Watts et al. 2003). The deformities result from physiological disruption during molting. Meregalli et al. (2001) found that sublethal concentrations of 4-*n*-nonylphenol, a known endocrine disruptor, caused mouthpart deformities in *Chironomus riparius*. Relatively low concentrations of 17α-ethinylestradiol and bisphenol-A (10 ng/L) caused mouthpart deformities. Typically, deformities include, loss, addition, fusing, and splitting and these are assigned a numerical score. When more than one deformity was observed, the scores were summed to provide an overall total score. Deformities were assessed relative to the normal arrangement of each of three structures (mandibles, the mentum, and the ephipharyngeal pectin). Observations were made by using a scanning electron microscope, and the most notable deformities were associated with the mentum. In the same study molting in response to these chemicals was also recorded and showed no statistically significant differences at the higher concentrations. This difference in endpoint sensitivity highlights the need to select multiple endpoints for assessment of potential EDCs and the need to understand the mechanism involved in selected effects noted for one mouth part over others such that screening tools could be developed for compounds such as EDCS that may demonstrate a specific deformity in response to those contaminants. Despite the similarities in

molting physiology between insects and crustaceans, it does not appear that morphology has been widely considered as an appropriate and measurable endpoint in crustaceans toxicological studies. A laboratory study showed that exposure to cadmium resulted in the development of abnormal genitalia in male *A. bahia* and malformed carapaces in males and females (Gentile et al. 1982). The time to the first appearance of the abnormalities was positively related to cadmium concentration. One study based on field-collected data found that four populations of *N. integer* contained many individuals with abnormal telson morphology (Mees et al. 1995). Such abnormalities could arise through physiological perturbations that occur during molting, and they could provide a quantifiable measure of disruption in endocrine-related functions.

180. The determination of the degree of fluctuating asymmetry (FA) found among mysids could provide a quantifiable, repeatable measure of morphological perturbations resulting from exposure to possible EDCs. FA is the asymmetric development of a normally symmetrical bilateral structure in which there is no tendency for one side of the structure to have a larger value than the other (Palmer and Strobeck 1986, Palmer 1994, Leung et al. 2000). FA is thought to arise because of environmental or genetic stress during development (Clarke 1993, Leung and Forbes 1996) and may result from a shift in metabolic energy from systems that maintain developmental stability to those that help organism compensate for increased stress (Sommer 1996). Measurement of FA, once appropriate characters have been determined, is relatively easy and requires only use of a microscope. Characters that have been measured are readily observable and include midge larvae teeth (*Chironomus*, Clarke 1993), copepod body spines (*Tisbe*, Clarke 1992), and shrimp antennae (*Palaemon*, Clarke 1993). However, Leung et al. (2000) mentioned that the measurement of single characters does not always reliably indicate environmental stress, and they suggested that an approach involving the use of composite indices of FA could increase the probability of detecting environmental stress. Although some studies of FA have been done on crustaceans (e.g., Clarke 1992, 1993), none has focused on mysids. Before FA could be used to detect mysid developmental abnormalities resulting from exposure to EDCs, preliminary studies examining several suites of potential characters would have to be performed.

181. Laufer et al. (2002), using male spider crabs *Libinia emarginata*, showed that ecdysteroids and low methyl farnesoate concentration (controlled by standard eyestalk ablation experiments) promoted allometric growth (disproportionate growth of body parts; for example, propodus of male spider crabs becomes disproportionately larger then the carapace during reproduction) while ecdysone and higher concentrations of methyl farnesoate inhibited allometric growth. Again, researchers need to understand the mechanistic causes before these changes can be attributed to effects.

182. The use of histology for assessing impacts associated with EDCs has been largely unexplored as an endpoint for crustaceans. One study, using the amphipod *Gammarus pulex,* examined impacts associated with a sewage treatment plant. A highly significant number of females collected displayed abnormal structure of oocytes in vitellogenesis. Particularly noted were uneven ooplasma, few yolk bodies, and lipid globules that were present and unevenly distributed. In addition, both the males and females had decreased body size possibly related to interference with the ecdysteroid receptor, which could have impacts on the molt cycle and interfere with vitellogenesis (Gross et al. 2001).

6.1.3 Behavior

183. The disruption of crustacean swimming behavior is one endpoint that has recently been investigated, particularly in mysid, as potentially informative in documenting sublethal exposure to contaminants. In a series of studies, Roast et al. (1998, 2000a, 2000b, 2001) determined that sublethal concentrations of cadmium and chlorpyrifos (an organophosphorus pesticide) significantly altered the swimming behavior of *N. integer*. Perturbations included decreased ability to swim against a current, an increase in general activity with no improvement in ability to swim against the current, and a reduced

tendency to maintain a position near the bottom. These changes in behavior could have important ecological impacts to the animals by causing them to be moved to an unfavorable habitat or by increasing their susceptibility to predation, thus indirectly resulting in a lethal response to contaminants at sublethal concentrations. However, if the disruption of swimming behavior is to be used as an endpoint in endocrine disruption studies, it should be applied with caution. Roast et al. speculated that the disruption of swimming that they observed was probably a nervous system effect related to interference with cholinergic pathways. Additionally, the time required to make behavioural observations is very long, making such endpoint very labour intensive; it might not be considered worthwile for a regulatory test.

184. Reduced feeding has been noted as a sublethal response to exposure to some contaminants. For example, Nimmo et al. (1981) noted, but did not quantify, reduced mysid feeding in response to exposure to some pesticides. Although difficult to accurately quantify, reduced feeding no doubt is an important sublethal effect of contaminant exposure that may be expressed in many of the life cycle parameters described below. Reduced feeding could lead to reduced growth, reduced time to maturity, and reduced egg production, among other factors.

185. One study using the amphipod *Gammarus pulex* looked at the combined effects of water and dietary uptake of 4-nonylphenol. Most feeding studies usually focus on one route of exposure and assume a constant feeding rate during the course of the study. The exposure route for benthic invertebrates includes direct contact with the sediment and pore water, uptake from the water column, and ingestion of food and other particles. 4-nonylphenol is more likely to be absorbed to the sediment particles because of its hydrophobic nature. The results of this study showed that feeding rates can be determined in a variety of ways depending upon how the data are analyzed (variable feeding rate versus mean feeding rate). The study showed that significant uptake of 4-nonylphenol occurred from food.

186. Schmitt and Seuront (2001) performed a 3D copepod trajectory using *Temora longicornis*, recorded in the laboratory. They observed a so-called multifractal random walk, which was considered as an inborn behavior, and not induced by uneven distribution of phytoplankton. They also identified three different modes of swimming, which may reflect the effect of gravity, predator avoidance, and feeding switch between two kinds of different food sources.

6.2 MEASURES OF REPRODUCTIVE PERFORMANCE

187. There are several measures of reproductive performance that can be used to assess sublethal response. For example, sexual maturity, the time to first brood release, the time required for egg development, fecundity, and alterations in reproductive characteristics in populations have all been used as endpoints. Zou and Fingerman (1997) showed that conditions of crowding, food shortage, and change in length of day could trigger a change from asexual to sexual reproduction, with males being produced initially. Females receive environmental cues, which trigger the ovaries to lay male-producing eggs. Exogenous agents may interfere with male differentiation and may affect this process of environmental cues to the ovaries.

6.2.1 Sexual Maturity

188. Khan et al. (1992) asserted that sexual maturity, which they described as the presence of gonads or a brood pouch, is a feasible endpoint for reproductive tests, because gonad maturation is essentially the first step toward reproductive output. Maturity allows for the measurement of effects to both males and females. Khan et al. used a dissection microscope to examine live mysids for the presence of gonads at the test termination. They quantified maturity as the ratio of the number of sexually mature mysids to the number of surviving mysids in each test replicate, and demonstrated that maturity was a sensitive endpoint for tests of 96-h and 7-day duration that were initiated with 8-day-old mysids. Others used the time

required for mysids to reach sexual maturity as a test endpoint (e.g., Gentile et al. 1983, Lussier et al. 1985). Gentile et al. (1983) used the development of testes or the presence of eggs in the oviduct to determine when sexual maturity was reached in male and female mysids, respectively. They reported that exposure to high levels of mercury significantly lengthened the time required for mysids to reach maturity, which then was expressed as delay in the appearance of eggs in the brood pouch and the release of young.

189. Exposure of *H. azteca* to 17α-ethinylestradiol showed several reproductive and morphological effects including: smaller gnathopods in females; skewed sex ratios favoring females; histological aberrations of the reproductive system, such as indications of hermaphroditism, disturbed maturation of germ cells; and disturbed spermatogenesis of the post F1 generation males (Vandenbergh et al. 2003). For both gnathopod growth and secondary sex characteristics, more pronounced effects were noted at the lower concentration (U-shaped curve), which may suggest a receptor-mediated response. It could be that 17α-ethinylestradiol causes disturbance of the androgen gland through interaction with the AGH or hormone metabolizing enzymes with subsequent changes in AGH activity.

6.2.2 Time to First Brood Release

190. In uncontaminated systems, the length of time to the release of the first brood is primarily related to environmental temperature, with some influence by salinity and an interaction between the two factors (McKenney 1996). McKenney (1996) determined that the shortest time to release of the first brood for *A. bahia* was about 16 days at a temperature of 28ºC and a salinity of 28‰. The time to release of the first brood increases with decreasing temperature and salinity. Because this parameter is measured as the number of days from hatching of the mysids used in the test until they release their first brood, it can represent the expression of more than one factor, including the length of time it takes a mysid to reach sexual maturity and the time required for eggs to develop in the brood pouch before being released. Most contaminant effects are likely to lengthen the time to release of the first brood. Lussier et al. (1985) found that several metals (mercury, zinc, nickel) significantly increased the time to first brood release, whereas others (e.g., cadmium, copper, silver) did not. Gentile et al. (1983) found that very high levels of mercury caused mysids to abort eggs that had been deposited in the brood pouch.

191. The time to first brood release is also one of the main endpoints often used in copepod life cycle tests aiming at identifying effects of environmental pollutants with a suspected specific biological activity (e.g., Breitholtz and Bengtsson 2001; Chandler et al. 2004).

6.2.3 Egg Development Time

192. Egg development time is measured as the number of days between the first appearance of eggs in the brood pouch and the first release of juveniles. Gentile et al. (1983) found that mercury did not significantly affect the brood duration in mysids, although several other reproductive parameters were affected. Winkler and Greve (2002) calculated incubation time for *N. integer* as the difference between the date of laying eggs in the brood pouch and the date of release of the juveniles from the marsupium. Data were collected by removing females with fertilized eggs from culture jars and placing them in individual containers. Temperature affected the start of maturation. At 15°C, development was much shorter (1.5 months) than at 10°C (3 months). The reproduction rate of *N. integer* increased at higher temperatures because of declining incubation periods plus an increasing number of neonates released per brood (more juveniles released as the female gets larger). Also, at higher temperatures the number of released juveniles per brood was highly variable, most likely due to the successive oviposition of the females. Overall, *N. integer* had double the reproduction success of *P. flexuosus* because of the longer incubation times and release of fewer juveniles.

193. Similarly, in copepods, the mean number of days from brood sac extrusion to naupliar hatching has been used to study egg development time (Cary et al. 2004).

6.2.4 Brood Size (Fecundity)

194. Brood size can be measured as the number of eggs per brood (Khan et al. 1992) or as the number of young produced, expressed either as total young per female or as young produced per available female reproductive-day (e.g., Gentile et al. 1982, Lussier et al. 1985, Baldwin et al. 1995, Hutchinson et al. 1999, Breitholtz and Bengtsson 2001, Bejarano and Chandler 2003, Chandler et al. 2004). The latter measure may be used to normalize differences in the number of females available per test concentration (Gentile et al. 1982). The number of available female reproductive-days is calculated by multiplying the number mature females by the number of days survived. McKenney (1996) showed that the number of eggs in the first brood of mysids was related to salinity and temperature, and that the largest number of eggs was produced at a temperature of 25ºC and 31‰ salinity. Because it is an important measure of reproductive success, any reduction in brood size can be interpreted as an indication of reproductive toxicity (Khan et al. 1992). However, brood size is also directly related to female size. Therefore, reduced fecundity in response to exposure to EDCs needs to be carefully evaluated to distinguish direct interruption of reproductive processes from a simple reduction in growth. Khan et al. (1992) also stated that the use of fecundity without supporting parameters to indicate reproductive impairment is not advisable, because fecundity is labor-intensive to determine, requires trained personnel, and ignores toxic effects on males. The most likely effect of contaminants is a reduction in fecundity (Hollister et al. 1980, Lussier et al. 1985), which in some cases is the only response to contaminant exposure (Lussier et al. 1999). Contaminant exposure can also result in the abortion of broods (Gentile et al. 1983). Lussier et al. (1999) reported, but could not explain, a seasonal difference in fecundity: more eggs were produced in the fall than in winter or summer. This phenomenon should be considered when comparing tests conducted at different times of the year. Lussier et al. (1999) concluded that fecundity was nonetheless a sensitive and useful endpoint.

6.2.5 Intersexuality and Sex Determination

195. Exposure to EDCs can result in profound alterations in the reproductive characteristics of populations, expressed as physiological or morphological changes in individuals. For example, the most commonly reported phenomenon is a condition, pseudohermaphroditism, in which female mollusks develop male reproductive structures in response to exposure to tributyltin (LeBlanc et al. 1999). Among the Crustacea, cases in which individuals showed intersexuality have been reported for several different taxa (see references cited in LeBlanc et al. 1999). Mees et al. (1995) reported intersexuality in natural populations of *N. integer* collected in northern Europe. Ford et al. (2004a, 2004b) found intersexuality in males and females of *Echinogammarus marinus*, an estuarine amphipod. Ford et al. (2004a) found that the polluted sites had a higher incidence of intersexuality than reference sites in Scotland. When compared to “normal” individuals, intersex amphipods matured more slowly and showed reduced fertility and fecundity (Ford et al. 2004b). Reduced fitness was attributed to difficulties in mating resulting from the larger size of intersex individuals and the abnormal morphology associated with the condition. However, links between such phenomena in crustaceans and EDCs have not been established (LeBlanc et al. 1999, Ford et al. 2004a, 2004b). Regardless, Ford et al. (2004a) identified a distinctive morphometry associated with intersex males and suggested that it might be a useful biomarker of endocrine disruption. In some cases, abnormal sex ratios could be the result of EDC exposure, as has been seen in wild populations of copepods (Moore and Stevenson 1991, 1994). Another field observation was made by Bareau and Grecian (2003), who observed intersexuality in the amphipod *Corophium volutator* (Pallas) at four sites of the Bay of Fundy, Canada. They found that intersex individuals had both male and female sexual appendages, e.g. the second antenna (which is longer in males than in females) was of intermediate appearance, and that no intersex individuals carried eggs. The origin of the intersexuality in this study was unknown. Further,

studies of daphnids have demonstrated that the population sex ratios found under good environmental conditions may be altered by juvenile hormone analogs (Olmstead and LeBlanc 2002, Tatarazako et al. (2003). Daphnids reproduce parthenogenetically, producing female young when environmental conditions are favorable, but produce male offspring when conditions become unfavorable. Five juvenile hormone analogs were shown to alter normal sex ratios in *D. magna* by stimulating the production of males (Tatarazako et al. 2003).

196. Relatively few field studies have been conducted addressing endocrine disruption to organisms found in estuaries, which have been shown to contain a variety of contaminants including sewage, chlorinated hydrocarbons, metals, and radioactive materials. Some of these contaminants have endocrine disruption potential (e.g., sewage effluents containing steroidal estrogens, DDT and its metabolites, and TBT) (Oberdörster and Cheek, 2001).

7.0 DESCRIPTION OF POTENTIAL MODES OF ACTION REFLECTIVE OF REPRODUCTIVE AND DEVELOPMENTAL IMPAIRMENT

7.1 RESPONSE TO ECDYSTEROID AGONISTS AND ANTAGONISTS

197. Concern has often been expressed in recent years about the disruption of endocrine systems in aquatic organisms by the action of organic and inorganic contaminants (e.g., Snyder and Mulder 2001; Depledge and Billinghurst 1999; Fingerman 1997). In a review by Hutchinson et al. (1999a), it was suggested that based on estimated figures, the concentration of EDCs expected to be introduced to the United States' aquatic environment could be as high as 2.16 ng/L for 17α-ethinylestradiol-derived contraceptives, and 41.5 ng/L for conjugated estrogens used in hormone-replacement therapy. Although there has been considerable research conducted on the health of fish exposed to EDCs, there is little information available for crustaceans (Baldwin et al. 2001, Hutchinson et al. 1999a, b).

198. Developmental abnormalities and toxicity to daphnid embryos were noted (at levels far below concentrations causing toxicity to maternal organisms) when the maternal organisms were continuously exposed and also when the embryos were collected from unexposed parents and exposed directly to testosterone. These developmental abnormalities and delays in molt frequency of neonates were mitigated when the daphnids were co-exposed to 20-hyrdroxyecdysone. These findings suggest that testosterone may function as an anti-ecdysteroid in crustaceans (Mu and LeBlanc 2002a). This study suggests that testosterone was able to block the activity of 20-hydroxyecdysone when both steroids were provided. Ecdysteroids for the early embryo stage originate from the mother and are transferred to the egg. Ecdysteroids present in the late embryo are in part synthesized from the embryo. Thus, when the embryo is exposed to an anti-ecdysteroid antagonist it could affect both pools (maternal and embryo) of ecdysteroids. This could be manifested by early- and late-stage abnormalities. When direct exposure of embryos is conducted, it would have no impact on the maternal pool of ecdysteroid resident in the embryo, but it would affect production of ecdysteroids within the embryo itself, most likely noted by late-stage developmental abnormalities.

199. In the environment most chemicals are present as mixtures, yet little research has been conducted to evaluate the potential endocrine disruption of complex mixtures. Toward that end, the toxicity of a chemical mixture of fenarimol and testosterone was evaluated using *Daphnia magna* to ascertain if combined exposure would result in greater than additive toxicity. By itself, fenarimol causes late-stage developmental abnormalities in embryos while testosterone interferes with early and late-stage embryo abnormalities (Mu and LeBlanc 2004). Fenarimol is a known ecdysteroid synthesis inhibitor of endogenous hormones; when combined with testosterone, a known ecdysteroid antagonist, fenarimol effectively inhibited hormone synthesis, paving the way for testosterone to bind to ecdysteroid receptors. By exposing only embryos to fenarimol, this would result in perturbations in late embryo development since maternal ecdysteroids would be present and active, whereas exposure of testosterone to isolated embryos would cause both early- and late-stage developmental abnormalities. Results showed that fenarimol increased the toxicity of testosterone, while testosterone had no effect on the toxicity of fenarimol. Additionally, a model was used to predict combined effects using algorithms. The predictive model was very effective in estimating the joint toxicity of these compounds for the independent action and synergy of both compounds. Further studies are needed to evaluate the role of complex mixtures on crustacean endocrine systems.

200. The endocrine and reproductive effects of EDCs mimic the effects of natural hormones, antagonize the effects of hormones, alter the pattern of synthesis and/or metabolism of hormones, and/or modify hormone receptor levels (Depledge and Billinghurst 1999). The ability of some environmental

contaminants to bind to steroid hormone receptors as agonists or antagonists in a recognized mechanism of toxicity to endocrine-related processes has been documented (LeBlanc and McLachlan 1999).

201. The literature is vague with respect to gender differences from exposure to ecdysteroids. Cuzin-Roudy and Saleuddin (1989) discussed possible differences in effects to male and female mysids, *Siriella armata*. This study showed that secondary vitellogenesis starts at the beginning of the molt cycle for this organism, when ecdysteroid levels are low. There is a striking difference between males and females at this point: in females, ecdysteroid levels were 10 times higher than those in males, but the response of the epidermis for molt preparation was the same. Females also had much higher levels of 20-E, ecdysone, and high polarity products, which are probably linked to the storage of ecdysteroids in oocytes during secondary vitellogenesis. Embryonic and post-embryonic development occurs in the marsupium of the females. Juveniles are released shortly before ecdysis, after which the adult female lays a new batch of eggs in the marsupium. A secondary vitellogenic cycle starts for a new batch of oocytes on the second day of the female molt cycle. Secondary vitellogenesis is strictly linked to the molt cycle. During development, gonads and gonoducts differentiate before the appearance of secondary sexual characteristics (Cuzin-Roudy and Saleuddin 1989).

202. Macrocrustaceans are in general fast-growing and slow-breeding organisms. Integration between molting and reproduction is a physiological necessity in females. Ecdysteroid, the chief hormone in molting, is thought to be involved with control of female reproductive activities. However, this is controversial. Investigations using amphipods have shown that levels of vitellogenin fluctuate with hemolymph ecdysteroid levels (see for example Cuzin-Roudy and Saleuddin 1989, Depledge and Billinghurst 1999).

203. In crustacean females, sequestered ecdysteroids may be passed on to the eggs for possible elimination and to function as morphogenetic hormones partaking in the control of embryogenesis and early development. The ovary in many crustaceans accumulates ecdysteroid for possible use during embryogenesis (Subramoniam 2000). Molting and reproduction are more evident in the female, because vitellogenesis is the central event of the female reproductive cycle along with secretion of a new cuticle during molting. Hormones play a role in the nutritive supply for molting and vitellogenesis. The ovaries eliminate ecdysteroids by forming ecdysonic acid as a necessary way to eliminate ecdysteroid in the eggs and embryos. They also form conjugates as a means of elimination. In embryos, there are concentrations of the three ecdysteroids – i.e., ecdysone, 20-E, and PoA – and their conjugates. There are fluctuations in embryonic ecdysteroids, as evidenced by one shrimp species, *Sicyonia ingentis,* in which the eggs after spawning contain low levels of ecdysteroid. The levels then rise through development, probably by the synthesis of this hormone by the embryo's Y-organ. The endogenous accumulation of ecdysteroid within the ovary is also known to function in the induction of meiotic maturation of the oocyte (Subramoniam 2000).

204. In one experiment, Subramoniam (2000) removed eggs from the pleopods of the freshwater prawn, *Macrobrachium nobilii*. The release of eggs quickened the next molting and reproductive cycle. In another experiment, Subramoniam (2000) found that although the ovarian cycle begins during the intermolt stage, vitellogenesis (serum levels) progresses into the next premolt stage. Premolt starts with the release of the larvae, and the next spawning occurs after ecdysis. Among penaeid shrimp, free spawning occurs during the premolt stage, followed by ecdysis (Subramoniam 2000). There are few *in vitro* studies available that focus on specific mechanisms involved in disruption in arthropods. The ecdysone receptor is in the same gene family as the thyroid receptor found in vertebrates.

205. In another experiment conducted by Bodar et al. (1990), daphnids were exposed to varying concentrations of cadmium and separately to exogenous ecdysone and 20-hydroxyecdysone to monitor any changes to the molt or reproductive cycle and to evaluate the role of ecdysteroids relative to molting and

reproduction. The study showed dose-dependent effects on molting and reproduction for the ecdysone and cadmium exposures. The effects of the higher ecdysteroid concentrations included unsuccessful exuviations, incomplete molting, and eventually death such that animals died before the age of potential reproduction was reached. The daphnids did not molt after treatment to ecdysteroids, and it was speculated that that they spent a disproportionate amount of energy on molting which negatively impacted the reproductive physiology.

206. For the cadmium exposures, a dose-dependent effect of cadmium on ecdysteroid titers was observed. At cadmium concentration of 5 μg/L there was an increase in levels above control (~210 pg ecdysone eq/mg dry weight) after 2-day exposure; these high levels declined after 8 days to levels approaching the control. As the cadmium concentration increased to 20 μg/L, there was a linear increase in ecdysone concentrations to around 750 pg ecdysone eq/mg, which is three times higher than controls. A pronounced decline in growth occurred under cadmium exposure. Also, a stimulatory effect on steroid hormones was seen such that increasing cadmium levels corresponded with increasing hormone titers. This stimulatory effect of cadmium on ecdysteroids has been observed for other organisms. Two theories are put forth: either the cadmium caused increased ecdysteroid levels which led to molt and reproductive impairment, or the cadmium interfered with the metal regulatory system through the metallothioneins and metalloenzymes that are involved in the molt cycle.

207. Incubation of ecdysteroid synthesis tissues *in vitro* is a method to detect endocrine modulators on molting hormone synthesis. This method was investigated as a potential biomarker using the midge *C. riparius.* This method can be used to determine if a particular chemical causes endocrine disruption at the sub-organismal level. Detailed methods for conducting ecdysteroid biosynthesis and subsequent measurement using radioimmunoassay techniques are described in Hahn and Schulz (2002). Male and female midges responded quite differently to this technique such that ecdysteroidogenic activity was significantly increased above controls for the males, while the opposite trend was found for the females. Also, exposed males developed faster than controls, whereas the treated females showed slower development than the controls. Even further, there is speculation that ecdysteroid metabolism is regulated by different processes in males and female midges during the fourth stage of larval development.

208. Block et al. (2003) used two crustaceans—a copepod (*Amphiascus tenuiremis*) and an amphipod (*Leptocheirus plumulosus*)—to determine ecdysteroid concentrations at different life stages. They also used a method known as fluorescence-based enzyme immunoassay (EIA) to quantify and compare ecdysteroid titers in such small organisms. Detailed methods for conducting the assay, including tissue collection and extraction of small sample volumes, is described. The overall synthesis, regulation, and metabolism of ecdysteroids used within and across species is most likely associated with variations in growth (molting), mating, and life cycles. Therefore, it is important to have a precise measure of ecdysteroid levels capable of detection at the femtomolar (10^{-15} molar) level.

209. In summary, detailed studies of crustacean response to ecdysteroids are lacking. Future studies that address sequence determination of vitellogenic genes and their hormonal activity could provide interesting insight into the vitellogenic process in this taxonomic group. The study of genomic response of ovarian maturation following ecdysteroid exposure deserves further attention and research. Synergistic and antagonistic actions of the X-organ sinus-molt and gonad-inhibiting neuropeptides, and the mandibular organ control over molting and reproduction are other areas requiring further research for use of crustaceans for EDC testing in the future.

7.2 RESPONSE TO VERTEBRATE-TYPE ANDROGENS

210. Vertebrate-type steroidal androgens have been measured in some crustaceans, but androgen receptors have not been documented. Presently, the androgenic gland has been identified only for

malacostracans (Block et al. 2003). Steroidal androgens can function directly as hormones in ways that do not require receptors, or they can be present as inactive components of steroid metabolic pathways (LeBlanc and McLachlan 1999). There is currently only limited published research that evaluates the androgenic hormones and their effect specifically on crustaceans. Administration of testosterone to shrimp has resulted in hypertrophy and hyperplasia of the androgenic gland. The androgenic gland is associated with the testis, and is responsible for the secretion of the androgenic hormone. This hormone is non-steroidal and is responsible for masculinization. Testosterone administered to shrimp and crab results in the increase in testis size and in the conversion from ovaries to testes in females (LeBlanc and McLachlan 1999). Experiments using *Daphnia magna* showed that testosterone, acting as an antagonist to 20-hydroxyecdysone, caused toxicity to neonates by interfering with the control of molting by ecdysteroids (Mu and LeBlanc 2002a). These studies, and the recent discovery of endogenous testosterone in *Neomysis integer* (Verslycke et al. 2002), suggested that studies designed to measure androgenic effects in crustaceans could be conducted. To date, an androgen receptor has not been identified nor cloned in crustaceans; research should be directed at identification and characterization of this receptor to aid in determining the usefulness of sex steroids as an evaluation tool for crustaceans (Verslycke et al. 2004a).

7.2.1 Endpoint Sensitivity

211. Vertebrate-type steroid hormones are found in the ovaries, testis, mandibular organ, and hemolymph of some crustaceans. Many of these steroid hormones exhibit fluctuations during gonadal development, suggesting a role in reproduction of crustaceans.

212. The identification of physiological targets of EDC in invertebrates is the approach taken by LeBlanc and McLachlan (1999). One example is that diethylstilbestrol and endosulfan have been shown to inhibit molting in immature daphnids, but to have no effect on the mature animals' fecundity. These effects may indicate that chemicals that are estrogenic to vertebrates could affect molting and reproduction in crustaceans, interfering with the proper function of the ecdysone receptor. In a study designed to examine antiandrogens, *Daphnia magna* was exposed to the compound cyproterone acetate, to determine whether it interferes with the androgen receptor as it does with vertebrates (LeBlanc and McLachlan (1999). The results showed impairment to growth. The exposed organisms were smaller, and there was a reduction in number of offspring. The latter was most likely due to the smaller size of the organisms, which would not have been able to accommodate a more normal number of brood in the pouch. The effects of steroidal androgens and chemicals that cause metabolic androgenization are consistent with interference to the delivery or packaging of nutrients into the developing eggs. Ecdysteroids, juvenoids, progestogen, and crustacean androgens have all been shown to influence vitellogenin or lipid production in arthropods. Androgens may interfere with one or more of the hormonally regulated processes that provide nutrients to embryos.

213. Baldwin (1997, 1998) conducted a series of experiments using the daphnid. During one experiment, the daphnid was exposed to 4NP, which resulted in changes in rates of elimination of testosterone and a corresponding decrease in glucose-conjugated testosterone, and an increase in the rate of production of various androgenic derivatives of testosterone. This is called metabolic androgenization, which is found to reduce fecundity of exposed daphnids associated with developmental abnormalities and high mortality of offspring. Results from a separate experiment with exposure to NP revealed no significant evidence of changes in steroid elimination processes, except at the highest concentration, which reduced elimination of glucose- and sulfate-conjugates and increased elimination of oxido-reduced derivatives. Effects were seen at sublethal levels for 4NP and approaching acute levels for nonylphenol. It has not yet been determined whether there is an androgen receptor in crustaceans; therefore, more studies are needed to determine the functional role of steroidal androgens.

214. It is possible that endogenous androgens may be precursors to other hormones, and that large doses of exogenously added androgens could elicit activity through other receptors. In crustaceans, testosterone is converted to androstenedione at various rates (LeBlanc and McLachlan 1999). Future studies may reveal that the conversion is affected by age, reproductive state, or photoperiod. It is possible that alteration in testosterone metabolism could serve as a biomarker, because effects are observed at concentrations less that those eliciting reproductive response.

215. Verslycke et al. (2003b) examined the elimination rates of testosterone by monitoring a specific set of metabolites (polar hydroxylated, nonpolar oxido-reduced, and glucose- and sulfate-conjugated). Various theories surround imposex of neogastropods exposed to TBT: inhibition of the cytochrome P450 system; blocking phase II sulfate conjugation; and interference directly with neurohormonal system, leading to changes in steroid titers. Further work is needed to confirm which mechanism(s) is responsible.

7.2.2 Gender Differences

216. Currently, there is no documented research that discerns gender differences in mysids or other crustaceans as a result of androgenic-type hormone response. Detailed mechanistic and anatomical studies would need to be conducted on mysids to ascertain whether differences in gender relative to EDCs can be observed.

217. In the 1980s, the condition of imposex (the imposition of male sex organs including a penis and vas deferens) was observed with increasing frequency on marine gastropods exposed to tributyltin (TBT) (Depledge and Billinghurst 1999). The mode of action of TBT giving rise to imposex is currently under investigation. Female snails exposed to TBT have elevated testosterone in the hemolymph, and injections of TBT into females induced penis formation (Depledge and Billinghurst 1999). Lee (1991) thought that many of the observed effects in mollusks are related to enzymes involved in TBT metabolism. Inhibition of a cytochrome P450-dependent aromatase (which normally converts 17α-estradiol to testosterone) could result in the accumulation of testosterone, which would otherwise be metabolized.

218. Studies of the shrimp, *Palaemon serratus,* showed that eyestalk ablation resulted in rapid maturation of the ovaries (reviewed by Fingerman et al. 1998). It was later shown that this effect is caused by the sinus gland containing a gonad-inhibiting hormone (GIH). This system is present in male crustaceans as well, and eyestalk ablation to induce gonadal maturation is a common practice on shrimp farms worldwide (Fingerman et al. 1998). The presence of gonad-stimulating hormones (GSH) was demonstrated in decapod crustaceans. In female crustaceans, the GIH and GSH acted directly on ovaries, which then secreted the ovarian hormone. Ovaries are a source of ovarian hormone, which induces the development of secondary female sexual characteristics. In male crustaceans, GIH and GSH acted on the androgenic gland. Two experiments were conducted to determine the role of the androgen gland using *Macrobrachium rosenbergii*. When the androgen gland was removed, the male became feminized, and when the androgen gland was implanted into a female, the female became masculinized (Fingerman et al. 1998).

219. In their review, Fingerman et al. (1998) reported that parasitism of crustaceans by rhizocephalans induced castration. The castration of the males often involved additional impairment to testicular function by modification of the secondary sexual characteristics, causing the males to take on female appearance. For example, the narrow male abdomen of crabs became wider, resembling that of a female. Several authors, such as Fingerman et al. (1998) have reported that in male shore crabs, *Carcinus maenas*, spermatogenesis occurred nonetheless in the testes of specimens found with feminized abdomens.

7.3 RESPONSE TO VERTEBRATE-TYPE ESTROGENS

220. Billinghurst et al. (1998, 2000, 2001) examined the effects of two estrogens, 4-*n*-nonylphenol and 17β-estradiol on larval settlement and the production of a larval storage protein (cypris major protein, CMP) in *Balanus amphitrite*. Cyprids use CMP during settlement and the early post-settlement development. Because CMP is structurally related to vitellin, which is analogous to vitellogenin, it can be used as a biomarker of estrogen exposure in lower vertebrates. The expectation in these studies was that cyprid settlement might be affected by the stimulation of CMP synthesis after larval exposure to environmental estrogens. The results of the 1998 study, however, showed reduced settlement after exposure to both estrogens, but that the cause was not related to endocrine disruption. The second study (Billinghurst et al. 2000) measured levels of CMP and found that they were elevated after exposure of nauplii to low levels of the estrogens. The third study (2001) measured effects of 4-*n*-nonylphenol and 17β-estradiol to larvae of *Eliminius modestus* (nauplii and cyprids). Specifically a disruption of the timing of larval development was noted, but this disruption was not consistent for different trials of this same experiment. This is in contrast to the 1998 study, but the studies were carried out at different times of year and with species that have different reproductive cycles. The variable response of different species to the same chemical reinforces the concept that development of larval crustaceans is subject to precise mechanisms and exposure to contaminants may depend on season and species. Further, this study showed that varying the timing of exposure of 4-*n*-nonylphenol and 17β-estradiol or the duration was critical. For example, organisms exposed for 12 months were significantly smaller than control organisms. As reported for other studies, Billinghurst et al. (2001) found that intermediate concentrations of NP are more disruptive than higher or lower concentrations.

221. Hutchinson et al. (1999a, 1999b) found that exposure to several steroids had no effect on the survival and development of copepod (*Tisbe battagliai*) nauplii and cautioned against extending the reported effects of steroid exposure in some species of crustaceans to the group as a whole. At about the same time, Bechmann (1999) showed that high levels (>62 μg/L) of nonylphenol were acutely toxic to *T. battagliai*, but that exposure to a low level (31 μg/L) did not affect any of the measured life-table parameters (survival, sex ratio, fecundity) measured. Breitholtz and Bengtsson (2001) did not find evidence of endocrine disruption in the harpacticoid copepod *Nitocra spinipes* after exposure to the estrogens 17β-estradiol, 17α-ethinylestradiol, and diethylstilbestrol. This contrasts with another study that exposed *Hyalella azteca* to 17α-ethinylestradiol at concentrations of 0.1 μg/L to 10 μg/L in a multigenerational experiment (Vandenbergh et al. 2003). Results showed that F1 males exposed from gametogenesis to adulthood developed significantly smaller second gnathopods; this response generated a U-shaped dose response curve suggesting a receptor-mediated response because effects were found at lower concentrations but masked at higher concentrations. Post F1-generation males exhibited histological aberrations of the reproductive tract (i.e., hermaphroditism, disturbed maturation of germ cells, and disturbed spermatogenesis); again these responses were more pronounced at the lower concentrations, suggesting a receptor-mediated response. Also noted, but not statistically significant, was that the populations exposed to 17α-ethinylestradiol for more than two generations tended to favor females.

222. Additional experiments conducted by Oberdörster et al. (2000) using *Palaemonetes pugio* in a 6-week pyrene exposure showed a significant increase in vitellin at 63 ppb and a significant increase in embryo mortality at 63 ppb. The increase in VTN could be a countermeasure against lipophilic compounds such as pyrene, because vitellins may be able to bind lipophilic compounds and transfer them to developing embryos.

223. In the shrimp, *Penaeus monodon*, 17α-estradiol and progesterone in free and conjugated forms increase in the ovary during vitellogenesis (Fairs et al. 1990). Metabolic precursors such as pregnenolone and dehydroepiandrosterone also increase and show a peak during the major vitellogenic stages, suggesting a pathway in crustaceans that is similar to that in vertebrates. Fairs et al. (1990) also reported that 17α-

estradiol and progesterone levels in the hemolymph showed fluctuations resembling that of serum vitellogenin levels during ovarian maturation. Estrogen could possibly control the stimulation of yolk synthesis, whereas the progesterone could control the prophasic meiotic maturation, causing germinal vesicle breakdown in the post vitellogenic oocytes. Exogenous injections of steroidal hormones induced vitellogenesis in the prawn, *P. japonicus*. In a study of a marine shrimp, *P. semisulcatus*, it has been shown that both the vitellogenin synthesis in the hepatopancreas and vitellin synthesis on the oocytes are coded by one gene (Subramoniam 2000).

224. Female crustaceans synthesize and secrete the protein vitellogenin into the hemolymph at the onset of oogenesis. There are a limited number of enzyme-linked immunosorbent assays (ELISA) that have been developed for measurement of vitellogenin or vitellin (VTN) in crustaceans but all are designed for detection in larger decapod species. A new method has been proposed by Volz and Chandler (2004) for measurement of VTN in small microquantities in a sediment-dwelling copepod, *Amphiascus tenuiremis.* This ELISA uses VTN-specific polyclonal antibodies from *Leptocheirus plumulosus,* which show specificity toward female copepod proteins. Quantities of purified VTN, used as a standard, were collected from grass shrimp eggs because it can be collected in relatively large quantities and it reacts well with anti-VTN antibodies from *L. plumulosus*. The results using this ELISA showed significant discrimination between gravid females and male samples. The final working range for the ELISA was from 31.25 to 1000 ng/mL with intra-assay and interassay CVs of 3.9 and 16.8%. Further, the detection limit was 2 ng/mL and the ability to quantify VTN in small numbers (four or more) copepods makes this ELISA a promising tool for further research for monitoring endocrine activity of toxicants to copepods and other crustaceans.

225. Studies (Pounds et al. 2002) to determine mode of action of several selected natural and synthetic steroids and xenoestrogens were examined using a combination of the *Tisbe battagliai* life-cycle test and also the B_{II} haemocyte line of *Drosophila melanogaster,* which is a screening assay to examine the agonist and antagonist effect of compounds to the cell line. One steroid tested, 20 HE, demonstrated reproductive effects on *Tisbe battagliai* at 26.9 μg/L and also demonstrated agonist activity to the ecdysteroid receptor, suggesting that the *in vivo* response was mediated via the receptor. The other steroids tested showed no response to either assay, indicating that even at high concentrations the synthetic and natural vertebrate steroids do not interact with the ecdysteroid receptor. Receptor-binding assays provide information relative to specific interaction of a compound to the endocrine system but do not give predictive information about how this compound will affect the whole organism.

7.4 RESPONSE TO OTHER HORMONAL DISTURBANCES

226. In their review paper, Fingerman et al. (1998) described other hormonal responses and disturbances in crustaceans, such as color-changing hormones, retinal pigment hormones, pericardial hormones, and blood glucose hormones. Each of these will be briefly described below, relative to crustaceans in general. Specific hormone disturbances to mysids as well as endpoint sensitivity and gender differences await further study.

227. The sinus gland is the storage and release site for color-change hormones, among others. The sinus gland is located proximal to the eye and lies next to the large hemolymph sinus (Fingerman 1997). For sessile crustaceans, the sinus gland is located in the head close to the optic centers. Investigators believe that 90% of axonal terminals that compose the sinus gland belong to neurons whose cell bodies lie in the medulla terminalis X-organ. Therefore, the medulla terminalis X-organ sinus-gland complex is similar to the vertebrate hypothalamo-neurohypophyseal complex (Fingerman 1997).

228. Color change is affected by cells called chromatophores, which are located in the integument. They are responsible for color change through their dispersion and aggregation. In an early experiment,

the hemolymph of a dark prawn specimen was transferred to a pale one (Fingerman 1997). When this organism was kept on a white background, it turned dark. The researcher then cut through the exoskeleton to sever any peripheral nerves that should innervate the chromatophores to determine whether color change was related to the endocrine system or to the nervous system. The incision had no effect on color change. Histological examination failed to show any innvervation of the chromatophores, which lead to the conclusion that color changes of this prawn are due to hemolymph-borne pigment concentrating substances.

229. For a variety of decapod crustaceans, chromatophores that cause integumentary color changes are controlled by antagonistically acting pigment-dispersing and pigment-concentrating neurohormones. For example, in the fiddler crab *Uca pugilator,* the neurohormone, 5-HT-serotonin triggers the release of red pigment-dispersing hormone, but has no effect on the black chromatophores (Fingerman 1997). The black chromatophores are triggered by norepinephrine, which releases a black pigment-dispersing hormone (BPDH). Studies have shown that the eyestalks of *U. pugilator* contained four times as much BPDH as did the control after exposure to naphthalene, due to naphthalene's inhibition of norepinephrine release. The opposite mechanism was observed for cadmium (Fingerman 1997). The eyestalks of control organisms contained three times more BPDH than did the cadmium-exposed crabs, which indicated that cadmium inhibited the synthesis of BPDH.

230. Retinal tissue contains pigments that control the amount of light striking the rhabdom (the photosensitive part of each ommatidium that composes the compound eye) through changes in position. Three types of retinal pigments have been categorized: the distal, proximal, and reflecting (Fingerman 1997). Migration of the distal pigment is controlled by the light-adapting hormone (LAH) and the dark-adapting hormone (DAH). Most studies of retinal pigments use the distal pigment, because techniques for its use are noninvasive. Several studies were conducted using *Palaemonetes vulgaris*. In one experiment conducted by Fingerman in 1959, this species was kept under constant illumination and then injected with extracts of eyestalks or sinus glands (described in Fingerman 1997). Because there is an initial light-adapted response followed by a dark-adapted response, Fingerman was able to induce a dark-adapting response from an organism kept under constant illumination, suggesting that eyestalks contain both LAH and DAH.

231. The sinus gland contains the source of CHH, which causes elevation of blood glucose levels for crustaceans. CHH is similar in structure to MIH. It has been determined that MIH and CHH also show similar activity (Fingerman 1997). Future studies should address the specific roles of these hormones in mysids and other crustaceans. Exposure of the freshwater prawn *Macrobrachium kistnensis*, and several species of crabs, to cadmium caused hyperglycemia. Similarly, exposure of *U. pugilator* to naphthalene caused hyperglycemia, although the mode of action is apparently different for the two compounds. Cadmium inhibits CHH synthesis, whereas naphthalene stimulates CHH synthesis; 5-HT apparently triggers release of CHH (Fingerman 1997).

232. Pericardial organs lie in the venous sinus that surrounds the heart, and the axon terminals could be part of the neuroendocrine system that releases hormones affecting the heart. Experiments showed that the hearts of three species, *Cancer pagurus, Homarus vulgaris,* and *Squilla mantis* responded to pericardial organ extracts with increase in both frequency and amplitude of the heart beat. Efforts to identify substances in the pericardial organs have revealed 5-HT, dopamine, and octopamine (Fingerman 1997).

7.5 HOW TO CONFIRM MODES OF ACTION – THE USE OF BIOCHEMICAL MEASURES

233. A number of biochemical measures are explored below as possible endpoints for EDC-exposure tests: metabolic disruption, steroid metabolism, vitellogenin induction, hormone levels, RNA:DNA ratios, protein levels, and the levels of cytochrome P450 enzymes and of blood glucose. Care must be taken when interpreting results of bioenergetic biomarkers such as those mentioned here, because many abiotic as well as toxic stressors affect metabolic processes.

7.5.1 Metabolic Disruption (O:N ratios)

234. Many of the perturbations expressed in the parameters described in Sections 4.2.4 could be related to changes in energy pathways resulting from chronic exposure to contaminants. McKenney (1985, 1989) and McKenney et al. (1991) showed that exposure to contaminants caused increased respiration rates in juvenile mysids, often with as little as 24-h exposure. The increased general metabolic demands related to contaminant exposure reduced growth by decreasing the amount of energy available to produce new somatic tissues. Normally, energy allocations to metabolism, growth, and reproduction occur, so that if there are changes in allocations of energy this may be an indicator of stress attributable to a toxicant or an environmental change (e.g., temperature, oxygen levels) (Verslycke et al. 2004a). It is important that bioenergetic endpoints include a baseline assessment of what is considered normal for crustacea. Further, alterations to energy metabolism can be assessed by using tools like the cellular energy allocation assay. Changes in metabolic usage can also be monitored using oxygen and nitrogen ratios, protein and lipid content, or the carbon to nitrogen ratios.

235. One way of predicting effects of abiotic stress on energy metabolism is the cellular energy allocation (CEA) assay. Verslycke et al. (2003b) conducted a study using *Neomysis integer* exposed to varying concentrations of tributyltin chloride (10, 100, and 1000 ng TBT Cl/L). Effects were noted using the CEA assay such as less overall consumption of energy and lower respiration rates than control mysids. These results show that TBT interferes with the energy metabolism by disrupting energy production process. Overall there was a loss of protein, lipids, and sugars in the organisms exposed to higher concentrations of TBT versus the control. Later, Verslycke et al. (2004b) showed that exposure to sublethal doses of nonylphenol and methoprene significantly increased energy consumption, resulting in less energy available for reproduction and growth. Using the CEA assay to assess the energy budget quantitatively for a particular organism helps to elucidate potential modes of action for toxicants. Methods for CEA measurements include spectrophotometric measures of lipid, protein, and sugar (Verslycke and Janssen 2002). Additionally, electron transport activities were measured by using INT (p-iodonitrotetrazolium violet) as an electron acceptor mimic in the electron transport system, which can provide a measure of oxygen consumption (Owens and King 1975 as cited by Verslycke and Janssen 2002).

236. Increased metabolic demands caused by exposure to contaminants could also impair mysid growth and reproductive capability. Exposure to relatively high concentrations (0.072 μg/L, 0.1 μg/L) of the pesticide chlorpyrifos caused increased oxygen consumption rates and reduced egestion rates in *Neomysis integer* (Roast et al. 1999). These responses resulted in reduced energy available for growth (i.e., lower "scope for growth"). Young mysids typically use high-energy lipids to meet metabolic demands, but change to metabolize proteins as they mature, thereby leaving more lipid material available for the production of gametes (McKenney 1989). Gorokhova and Hansson (2000) found that a 6% decrease in the carbon:nitrogen ratios occurred as juvenile *Mysis mixta* matured to gravid adults and asserted that this was evidence that maturation and reproduction are fueled primarily by lipids. Increased metabolic demands caused by exposure to contaminants is met by greater lipid metabolism, which reduces the lipids available to meet reproductive needs (McKenney 1985, 1989). These changes in metabolic

substrate usage can be measured by monitoring the oxygen : nitrogen (O:N) ratio of test organisms. The O:N ratio indicates the relationship between the amount of oxygen consumed by an organism to the amount of nitrogen excreted, and shows the relative role protein catabolism plays in the organism's energy budget (Carr et al. 1985; McKenney 1985). *Americamysis bahia* showed a change toward lipid metabolism after only 4 days of exposure to high concentrations of cadmium (Carr et al. 1985). Mysids showed increased metabolic demands after four days of exposure to the herbicide thiobencarb (McKenney 1985) or five days of exposure to the defoliant DEF (McKenney et al. 1991). High O:N ratios occurred among maturing mysids exposed to the compound, indicating a shift to lipid metabolism that would have reduced the lipids available for gamete production. All three studies concluded that changes the O:N ratio measured among test mysids was a sensitive indicator that could provide for the relatively early detection of reproductive impacts by contaminants.

7.5.2 Steroid Metabolism

237. Although the role estrogens play in crustacean reproduction is not known (Baldwin et al. 1995), these steroids are important in other invertebrate groups. Because of the likelihood that crustaceans could be exposed to environmental estrogens such as dichlorodiphenyl trichloroethane (DDT), polychlorinated biphenyls (PCBs), and nonylphenols (nonionic surfactants), there is the potential for these compounds to disrupt steroid metabolism. Baldwin et al. (1995, 1997, 1998), studied the effects of three environmental estrogens, diethylstilbestrol (DES), 4-nonylphenol (4NP), and nonylphenol polyethoxylate (NPPE), on the steroid metabolism of the freshwater daphnid, *Daphnia magna*. Their work focused on the disruption of the metabolic elimination of testosterone after short- and long-term exposure to the test compounds and sought to determine whether such an analysis could be used as an early indication of reproductive impairment. They measured differences in the glucose conjugation, sulfate conjugation, hydroxylated and reduced/dehydrogenated metabolites of ^{14}C-labeled testosterone in daphnids exposed to sublethal concentrations of the test compounds. They found that the different compounds had different effects on testosterone metabolism. For example, DES increased glucose conjugation, but did not affect sulfate conjugation, whereas 4NP reduced both of these elimination processes. In their two earlier studies (Baldwin et al. 1995, 1997), Baldwin's group proposed that changes in testosterone metabolism could provide an early indication of potential reproductive toxicity after sublethal exposure to suspected EDCs. However, in their 1998 paper, Baldwin et al. studied NNPE, a nonionic surfactant that degrades to nonylphenol, and did not find significant disruption of steroid metabolism after short-term exposure. They did report some effects after chronic exposure and postulated that those could have resulted from the degradation of NPPE to NP. Therefore, they cautioned that use of short-term exposures as an early warning indicator might underestimate chronic effects resulting from bioaccumulation and bioactivation of the test compounds.

238. Verslycke et al. (2002) studied testosterone metabolism in *Neomysis integer*. Significantly, they detected endogenously-produced testosterone in male and female mysids. They also found an anabolic steroid, β-boldenone—the first known occurrence of the compound in an invertebrate—in mysids exposed to testosterone added to the test medium, although the metabolic pathway by which it is formed was not discovered. A vertebrate estrogen, 17α-estradiol was not detected. Verslycke et al. (2002) showed that testosterone metabolism in mysids involves phase I (oxido-reduced/hydroxylated) and phase II (conjugated) derivatives. The study proposed that changes in invertebrate steroid metabolism might be used to indicate exposure to EDCs. To evaluate this idea, Verslycke et al. (2003b) examined the effect of exposure to tributyltin on the elimination of testosterone by the mysids. The authors summarized the effect of TBT exposure on testosterone elimination by calculating the metabolic androgenization ratio—the ratio of the eliminated oxido-reduced products to the polar products (hydroxylated plus conjugated). TBT exposure changed testosterone metabolism by inducing reductase activity at low, but not high, concentrations and reducing sulfate conjugation, although the mechanisms by which these occurred were not identified. To further refine the idea that changes in testosterone metabolism could be used to indicate

exposure to an EDC, Verslycke et al. (2004b) subjected individuals of *N. integer* to seven compounds (testosterone, flutamide, 17*α*-ethinestradiol, precocene, nonylphenol, fenoxycarb, methoprene) suspected of having endocrine disruption properties. All seven were very toxic to *N. integer* with 96-h LC_{50} values ranging from 0.32 mg/L (methoprene) to 1.95 mg/L (testosterone). The effects of sublethal doses of nonylphenol and methoprene on testosterone metabolism were investigated and found to be significant. Methoprene and nonylphenol affected phase I testosterone metabolism in a manner similar to that of TBT (induced reductase activity at low concentrations of the chemical). The two compounds had opposite effects on phase II metabolism. Glycosylation was increased at a high (100 µg/L) dose of nonylphenol, but was significantly reduced at the same dose of methoprene. The metabolic androgenization ratio showed a dose-dependant increase after exposure to methoprene, but after exposure to nonylphenol was significantly increased only at the lowest concentration tested, i.e., 10 ng/L.

7.5.3 Vitellogenin

239. Depledge (unpublished, cited in Depledge and Billinghurst 1999) found that exposure to 4-n-nonylphenol induced the production of vitellogenin in decapods. Vitellogenin production is most likely controlled by the hormones primarily involved in molting, although in some crustacean groups this is likely not the case (Subramoniam 2000). The molt-inhibiting hormone and 20-hydroxyecdysone (20HE) are involved in the production and regulation of vitellins and there are several feedback loops (positive and negative) involved. To date, because of the complexity of vitellogenesis, it has not been used as a biomarker of endocrine disruption (Oberdörster and Cheek 2001). Little research has been conducted on vitellin expression in crustaceans, possibly due to the lack of antibodies, which have been shown to have cross-reactivity with other species. Most often polyclonal antibodies are used and available for crustaceans with the drawback that these antibodies may cross-react with other nonvitellin proteins if developed from impure vitellogenin. One such study, using grass shrimp exposed to pyrene, showed up-regulation of vitellin and, when the females were transferred to clean seawater, there was an increase in embryo mortality, suggesting that the vitellin can transport lipophilic contaminants to developing embryos.

240. As pointed out by Verslycke et al. (2004a), to understand the potential effects of xenobiotics on reproduction, there must be accurate measures of vitellogenin and vitellin in crustaceans. A few studies have been done to characterize and purify vitellin (e.g., Volz et al. 2002), and to develop vitellin ELISAs (e.g., Volz and Chandler 2004). Additional studies need to be conducted to evaluate the utility of such techniques for ED evaluation.

7.5.4 Hormone levels

241. Since crustaceans need to molt to grow and achieve a full adult body size (Charmantier et al. 1997, Chang 1997), the crustacean body goes through several morphological and functional changes, life events which may be sensitive to disruption of chemical substances. As indicated above (section 7.1), Block et al. (2003) determine ecdysteroid concentrations at different life stages of the copepod (*Amphiascus tenuiremis*) and the amphipod (*Leptocheirus plumulosus*). A fluorescence-based enzyme immunoassay (EIA) was used to quantify and compare ecdysteroid titers in such small organisms as copepods. Ecdysteroids may also be detected by Radioimmunoassay (RIA), a method employing radioactive isotopes to label either an antigen or an antibody. The isotope emits gamma rays, which are usually measured following removal of unbound (free) radiolabel. This has for instance been reported by Chang and O´Connor (1979) and since then been used for e.g. amphipod ecdysteroid level determinations (Jacobson and Sundelin 2006). The detailed methods for conducting the assay, including tissue collection and extraction of small sample volumes, could be useful for ED evaluation also in other crustacean species.

242. Methyl farnesoate (MF) is the unepoxidated form of the insect juvenile hormone (JHIII) and is produced in the mandibular organ. Recent studies have suggested that MF functions in a similar manner in

crustaceans as JH does in insects (Laufer and Biggers 2001). MF is involved in reproductive maturation and in morphological changes most notable from late juvenile stages to adult. MF has high potential as a biomarker of chemical activity at the endocrine level, but it is extremely lipophilic, and difficult to isolate and assay routinely. Various investigators have conducted eyestalk ablation studies in which crustaceans such as shrimp, crayfish, and fiddler crabs were stimulated to reach ovarian maturation. JH analogs such as methoprene have inhibited early larval and post larval development in the shrimp *Palaemonetes pugio*; they actually enhanced premetamorphic stage development.

7.5.5 RNA:DNA ratios

243. Identification of biochemical changes, such as RNA contents, which are related to growth and metabolism (Dahlhoff 2004), can be used to determine if an organism has been exposed to a stress, including contaminants (Yang et al. 2002). The rationale is based on the fact that the RNA content of tissues or whole organisms consists primarily of ribosomal RNA (rRNA). Consequently, concentration of rRNA, at any given time is directly related to the protein synthesis of a cell (Elser et al. 2000). The quantity of RNA is directly linked to the growth of the individual (Saiz et al. 1998). For example, in small metazoans, which have high metabolic rates of biosynthesis, a high portion of RNA is required (Brown et al. 2004). DNA exists in a quasi-constant quantity in a somatic cell and therefore might be used as an index of the number of cells (Buckley et al. 1999). Based on this, it is becoming increasingly common to assess growth rates in a variety of aquatic animals by using the RNA:DNA ratio (e.g., lobsters: Rosa and Nunes 2003; krill: Cullen et al. 2003; copepods: Saiz et al. 1998, Campbell et al. 2001, Gorokhova 2003; daphniids: Vrede et al. 2002; cirripeds: Desai and Anil 2002). However, in small crustaceans, such as daphnids, copepods and decapod larvae, RNA content alone may be a more sensitive endpoint than the RNA:DNA ratio due to the growth- and ontogeny-related fluctuations in DNA content (Gorokhova and Kyle 2002, Rosa and Nunes 2003, Gorokhova 2003). Also, Dahl et al. (in press) found that in the harpacticoid copepod *Nitocra spinipes* exposed to the pharamaceutical substance Simvastatin, RNA levels correlated with regular life table endpoints (i.e. development rate).

7.5.6 Protein levels

244. Crustacean larvae show dramatic growth and morphogenetic changes, which obviously may be affected by environmental conditions or chemical substances. Studies on the cellular response to conditions of physical/chemical stress have played a significant role in diverse areas. Variations in protein contents may be due to stress factors, e.g. variations in environmental factors such as temperature (e.g. Verslycke and Janssen 2002). Verslycke and Jenssen (2002) found that a decline in protein content during a 4-day study in the mysid *N. integer* was caused by the ability of *N. integer* to use its protein content as a source during starvation or stress. Verslycke and Janssen (2002) also state that the exoskeleton of *N. integer* contains large amounts of proteins and consequently large variations in protein contents could be a result of molting. Torres et al. (2002) investigated the effects of reduced salinity on larvae of four crustacean speices (*Cancer pagurus*, *Homarus gammarus*, *Carcinus maenas*, *Chasmagnathus granulata*). They found that the protein contents were generally lower in the exposed groups in contrast to the control group. Some exposed groups even had lower protein contents than at the beginning of the experiment. When exposed to stress, an organism may react by producing stress proteins. Stress proteins are a ubiquitous family of proteins, present within cells at constitutive levels. Upon exposure to stress, they are induced in order to provide protection against cellular damage. These stress proteins interact with other cellular proteins; often damaged as a result of stress, and in doing so, minimize chances for these proteins to further engage in synthetic or regulatory processes (Feder and Hoffman 1999). Hence, protein levels are highly correlated to stress factors of an organism, and may therefore be a useful complement to more traditional ecotoxicological endpoints as well as other biochemical markers for ED evaluation.

7.5.7 Cytochrome P450 Enzymes

245. Cytochrome P450 enzymes (CYPs) are commonly occurring proteins that are involved in the metabolism (i.e., detoxification) of many exogenous and endogenous compounds (Snyder 2000, Snyder and Mulder 2001, Verslycke et al. 2004a). Snyder and Mulder (2001) measured CYP45 levels, a family of P450 proteins found in the lobster *Homarus americanus,* and thought they must be involved in the molting cycle, in response to exposure to the pesticide heptachlor, a known EDC. They found that peak levels of ecdysteroid hormones, and accordingly, molting, occurred later in heptachlor-exposed larvae than in control larvae, indicating that heptachlor disrupts steroid molting hormone metabolism. They also found CYP45 levels in lobster larvae exposed to heptachlor on Days 1, 2, or 3 after hatching to be 15 times higher than they were in those exposed to control solutions. Levels of CYP45 typically peaked 1 to 2 days after exposure, and then decreased. Snyder and Mulder suggested that it could be a useful early biomarker of exposure to EDCs, because it showed a dramatic and rapid increase in levels after exposure to heptachlor. In their testosterone metabolism study, Verslycke et al. (2002) showed that *Neomysis integer* has many P450 enzymes that comprise its complex steroid hydroxylation system. They suggested that changes in P450 activity could be used as a biomarker indicating exposure of this mysid to EDCs.

246. It is known that most P450 activities occur in the hepatopancreas, but other tissues, such as the gills, stomach, intestines, and antennal glands, have demonstrated some P450-like roles. Oxidative metabolism, most notably of PAHs, has been demonstrated in many crustacean species. A detailed review of studies conducted examining the metabolic changes (mostly caused by benzo(a)pyrene) in crustaceans can be found in James and Boyle (1998). This paper also offers suggestions of P450 involvement and or metabolism relative to drugs, steroids, and pesticides. Several studies have shown the presence of testosterone and progesterone in gonadal tissues and hemolymph. The source of these steroids is not well known but it is likely that several cytochrome P450-dependent steps are involved. Future directions should include genomic information on the crustacean P450 system, the definitive identification of an aH receptor, and a best understanding of the regulation of the P450 system, particularly for steroid synthesis.

247. Changes in *cytochrome P450 enzymes*, which function in the detoxification of many exogenous and endogenous compounds, may be associated with disruption of the hormonally regulated molting process and are therefore appropriate to measure in EDC studies. The general procedure for determining levels of cytochrome P450 in crustacea involves homogenization of whole animals, centrifugation, and the collection of the resulting supernatant. Quantification of the levels occurs via gel electrophoresis. Snyder and Mulder (2001) described a specific method for determining CYP45 (a family of CP450 enzymes) levels in daphnid crustaceans.

7.5.8 Glucose Levels

248. Levels of glucose in crustacean blood (i.e. haemolymph) are regulated by a hormone, crustacean hyperglycemic hormone (CHH), which is produced in the sinus gland (Fingerman et al. 1998). Release of CHH increases blood glucose levels. Some exogenous compounds have been shown to affect the levels of glucose in the bloodstreams of several crustacean taxa, probably be stimulating (e.g., naphthalene) or inhibiting (e.g., cadmium) CHH synthesis (Fingerman et al. 1998). Measurement of changes in blood glucose levels in crustacea exposed to potential EDCs could be indicative of hormonal perturbation other than that associated directly with reproduction or molting. Hyperglycemia is a common response to environmental or toxicant interactions, and changes in blood glucose levels in response to presence of EDCs may be an indication of interference with hormonal activities (Verslycke et al. 2004a). However, many abiotic and toxic responses affect energy metabolism, and researchers must be able to make distinctions between natural variations in hormone levels and variations that are caused by EDCs.

8.0 RESEARCH NEEDS AND OTHER CONSIDERATIONS

8.1 RESEARCH NEEDS

249. The last decade has witnessed several arthropod studies where e.g. vertebrate steroids or industrial pollutants with known endocrine potency in mammals have had little or no effects related to endocrine disruption (Caspers 1998; Bechmann 1999; Hutchinson et al. 1999a, b, Breitholtz and Bengtsson 2001). Although e.g. estrogens have been identified in crustaceans (e.g. Novak et al. 1990), their functional role has not been established. This is, however, not surprising since arthropods have endocrine systems that differ widely from the vertebrates' (e.g. Hasegawa et al., 1993; Subramoniam, 2000). It has therefore been recommend focusing on the unique invertebrate endocrine functions rather than having man's hormone system as a reference when carrying out ecotoxicological research to find potential endocrine disruptors in environmental risk assessments (Breitholtz et al. 2006). In fact, it is possible that systematic studies including endocrine irregularities in arthropods will reveal further effects by other substances than those that give effects in vertebrates.

250. In a recent review, Sumpter and Johnson (2005) have indicated ten major lessons from endocrine disruption in the aquatic environment, which we should learn from in our way forward. At least three lessons are of special interest for the current DRP. First, we need to find out what is normal before we can say what is abnormal. In other words, we need to obtain baseline information on e.g. endocrinology in those groups of species we wish to protect. Second, it must be clear that one animal's poison may not be another's. The challenge is to predict which groups are the most likely to be selectively targeted by a given substance. Third, the last decade has shown that acute toxicity testing cannot detect endocrine disruption. Clearly, chronic toxicity tests can give messages entirely different from that of acute toxicity. As a consequence, the authors highlight the work conducted within regulatory bodies, in particular OECD, to establish and validate new testing procedures. This illustrates the need for development and establishment of new arthropod life cycle guidelines, as has been discussed and highlighted in the current DRP.

251. Hutchinson (2002) has also recommended that for environmental assessments of potential invertebrate endocrine disrupters, attention should be given to the use of reference compounds to validate new invertebrate test methods, evaluating in vitro data from both mammalian-type and arthropod hormone receptor assays, utilize small-scale test methods which do not generate effluent handling problems, using relevant environmental concentrations during testing and considering using improved test methods for which molecular tools are available. In this context, the rapid progress in the field of genomics, i.e. the study of how an individual's entire genome translates into biological functions, is beginning to provide tools that may assist our understanding of how chemicals impact ecosystem health. The term ecotoxicogenomics has recently also been proposed to describe the integration of genomics and ecotoxicology (Snape et al. 2004). Moreover, since proteins direct biological processes, the physiological state of an individual organism is perhaps better reflected by the proteome than the genome. Recently, new computational tools have facilitated dramatic advances in proteomics, which provides a comprehensive and quantitative picture of protein expression and its changes in an organism. For example, proteomic tools have been successfully applied in several invertebrate species, including nematodes (Schrimpf et al. 2001) and fruit flies (Vierstraete et al. 2003). The development of proteomics in standard invertebrate species, such as *D. magna*, has been identified as being of major importance for future environmental assessment of chemicals (Snape et al. 2004). In fact, increased knowledge about protein expression and its changes upon toxic exposure will in itself facilitate the mechanistic understanding of the toxic response, including endocrine irregularities.

252. Cellular processes, such as protein expression, inevitably determine individual and population rates. Hence, the concerted study of possible links between effects at the cellular (i.e. proteomics), individual (i.e. life table variables such as growth and fecundity) and population level (i.e. predictions of growth rate by the use of life table data) may result in powerful tools to be used for environmental assessments of potential endocrine disruptors. Rotchell and Ostrander (2003) have also pointed at that these and other molecular techniques, which are routinely used in medical research to identify specific genes and proteins, also could be used in the search for endocrine disruptors among aquatic organisms. Such tools will meet demands of both mechanistic understanding and ecological relevance (Snape et al. 2004). Hopefully, if clear links may be established between different levels of biological organization, this would also facilitate extrapolations, from lower to higher order of levels, and vice versa. In this context, arthropod life cycle tests may be the optimal platform to conduct such extrapolations, which could substantially enhance the regulation of potential endocrine disruptors in the future.

253. Chemicals that interfere with the biosynthetic and/or biotransformation pathways of arthropod hormones (such as ecdysteroids) would consequently have impact on individual and population growth (Verslycke et al. 2002). Any progress in the development of molecular techniques (both *in vitro* and *in vivo*) that may help revealing modes of action of chemicals in arthropods should be welcomed. Hutchinson (2002) claims that such a mode-of-action approach in e.g. arthropods could have both cost-effective and ethical benefits. In this context, it would be necessary to develop biomarkers for the proposed species; promising biomarkers could be the induction of vitellogenesis in males, and the inhibition of aromatase in females (Depledge unpublished, cited in Depledge and Billinghurst 1999). As more evidence for endocrine disruption responses is gathered from experimental research, mechanistic studies would be required to determine the specific ways in which chemicals can disturb hormones. That is, it becomes important to distinguish between endocrine disruption and metabolic toxicity and to distinguish between primary and secondary effects. The final step in the strategy would be to conduct field surveys to detect and confirm that endocrine disruption effects actually occur *in situ* (Depledge and Billinghurst 1999).

8.2 REGULATORY CONSIDERATIONS

254. In the EU the legally binding standardized Testing Methods to determine the hazardous properties of chemicals are contained in Annex V of Dir 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances. They play a central role in the EU policy on chemicals control and they are referred to in many other pieces of EU legislation (e.g. those related to dangerous preparations, pesticides, cosmetics and biocides also refer to these methods). To have standardized Testing Methods for chemicals at the EU level is also important for ensuring the Single Market & Free Trade inside the Union because the free movement of goods between the Member States is based on the mutual acceptance of the risk evaluation made by each country and this, in turn, relays on the mutual acceptance of the data generated when testing the chemicals. Obviously the same can be said at a global level, for this reason, the work is closely linked and co-ordinated with the parallel OECD Test Guidelines Programme. In general, the EU adopts the OECD Test Guidelines principally unchanged. Today there are no internationally agreed (i.e. OECD, EU) test methods to determine the potential environmental effects of endocrine disrupters. Under the existing legislation within the EU, the assessment of the toxic potential of a chemical is dependent on the type of chemical. For example, pesticides and biocides have separate legislation governing their testing and assessment. This existing legislation, while accounting for detrimental endocrine related effects on reproduction or disease states such as cancer, does not use disruption to the endocrine system as an endpoint per se. However, in 1999 the European Commission adopted a Community Strategy for Endocrine Disrupters (COM(1999)706) containing a number of short- and long-term actions. Research and development of agreed test methods for endocrine disrupters are prioritized in the Strategy. Furthermore, in the proposal for a new EU regulatory framework for chemicals, REACH (Registration, Evaluation and Authorisation of Chemicals) an authorisation system for uses of substances and the placing on the market of substances for such uses is established for the substances of very high

concern. Substances of very high concern are defined as: substances that are category 1 and 2 carcinogens or mutagens; substances that are toxic to the reproductive system of category 1 and 2; substances that are persistent, bioaccumulative and toxic or very persistent and very bioaccumulative; and substances such as endocrine disrupters, which are demonstrated to be of equivalent concern. The authorisation provisions require those using or making available substances with properties of very high concern to apply for an authorisation of each use within deadlines set by the European Commission. The aim of the authorization is to ensure the good functioning of the internal market while assuring that the risks from substances of very high concern are properly controlled and that these substances are eventually replaced by suitable alternative substances or technologies where these are economically and technically viable. The proposed new EU legislation is expected to come into force in 2007.

255. Chemical substances in the United States are regulated under several statutes. Predominant among these for use in requesting data and collecting risk information include the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) for pesticide active ingredients, the Toxic Substances Control Act (TSCA) for general chemicals, the Federal Food, Drug ad Cosmetic Act (FFDCA) for food additives, pharmaceuticals and personal care products, and the Clean Water Act (CWA) for surface water and drinking water contaminants. Any of these authorities may be employed singly or in combination in obtaining hazard data relevant to potential endocrine disrupting substances. Existing test guidelines employed for aquatic invertebrates include various acute and chronic methods across several taxa, including daphnids, copepods, mysids, penaeids, chironomids, and pelecypods. The primary chronic methods for aquatic arthropods, which are routinely required for outdoor use pesticides, are the OPPTS 850.1300 *Daphnia* chronic toxicity test (which is consistent with the OECD TG 211) and the OPPTS 850.1350 Mysid chronic toxicity test (no OECD counterpart). Neither of these test methods is considered sufficient for fully addressing potential endocrine disrupting substances. As part of the Endocrine Disruptor Screening Program (EDSP), a two-generation mysid test procedure (described in Section 5.1.1.1 and Annex 1) is being developed to provide a fully comprehensive second tier evaluation of substances identified as possible endocrine disruptors in first tier studies. Although the focus of the EDSP tier one is on vertebrate relevant endocrine processes, the mysid test in tier two is deemed necessary to assess the potential adverse consequence of tier one identified putative disrupting agents to invertebrate endocrine regulated processes.

256. In Japan, chemical substances (including potential endocrine disruptors) are under the control of the amended Chemical Substances Control Law. This law concerns production, import and export of chemical substances. In spring 2002, the Ministry of the Environment also formed a task force on endocrine disrupters testing and assessment for invertebrates (crustaceans).

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ANNEXES

ANNEX 1 – Mysid life cycle draft protocol ***(available from the OECD upon request, please contact env.tgcontact@oecd.org)***

ANNEX 2 – Daphnia life cycle draft protocol ***(available from the OECD upon request, please contact env.tgcontact@oecd.org)***

ANNEX 3 – Copepod life cycle draft protocol ***(available from the OECD upon request, please contact env.tgcontact@oecd.org)***

ANNEX 4 – Chironomid life cycle draft protocol ***(available from the OECD upon request, please contact env.tgcontact@oecd.org)***