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# DETAILED REVIEW PAPER (DRP) ON MOLLUSCS LIFE-CYCLE TOXICITY TESTING

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# DETAILED REVIEW PAPER (DRP) ON MOLLUSCS LIFE-CYCLE TOXICITY TESTING

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

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## FOREWORD

This document presents a Detailed Review Paper (DRP) on Molluscs Life-Cycle Toxicity Testing, prepared jointly by Germany and the United Kingdom. It has been reviewed by OECD countries in 2009 and revised accordingly.

The draft DRP was approved by the Working Group of the National Coordinators of the Test Guidelines Programme at its meeting held on 23-25 March 2010. The Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology agreed to its declassification on 5 May 2010.

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

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**Executive Summary**


I. This draft Detailed Review Paper (DRP) was requested by OECD in 2008 and has been prepared by Germany and the United Kingdom. It is the result of collaborative efforts between scientists from the two countries, but has benefited from the advice of a much wider range of experts.

II. The purpose of the DRP is to review what is known about the responses of molluscs to endocrine disrupting chemicals (EDCs), to consider whether it would be desirable and feasible to standardise mollusc-based partial- and full-lifecycle tests that are sensitive to EDCs and to other chemicals, and if so, to recommend suitable methods for optimisation and validation.

III. The review concludes that there is a need for mollusc-based lifecycle tests, primarily because the only invertebrate tests of this type that are currently being internationally standardised use arthropods alone, yet mollusc species are ecologically and economically important, and are known to be uniquely sensitive to a number of EDCs (e.g. organotins) and other substances (e.g. copper). No other invertebrate phylum offers similar opportunities at the present time.

IV. Although there are some similarities between the endocrine systems of vertebrates and molluscs, the lack of knowledge about how EDCs act in molluscs currently prevents the use of mollusc-based tests as surrogates for tests with aquatic vertebrates such as fish.

V. Given our relatively poor understanding of molluscan endocrinology, it is not currently possible to recommend the development of mollusc-based screening tests for the identification of EDCs. On the other hand, we are now in a position to start standardising apical mollusc tests involving partial or full lifecycle exposures. In principle, these are expected to be responsive to any chemicals (EDCs or non-EDCs) with activity in molluscs, even though they may not provide diagnostic information on modes of action (MoA).

VI. The review recommends three apical test procedures for optimisation and possible validation. The first is a partial life cycle (PLC) test with the freshwater gastropod *Potamopyrgus antipodarum*, the second is a full life cycle (FLC) test with the freshwater pulmonate *Lymnaea stagnalis*, and the third is an FLC test with the marine bivalve *Crassostrea gigas*. It would also be straightforward and worthwhile to develop an FLC test protocol using *P. antipodarum* and a PLC protocol using *L. stagnalis*.

VII. There is more experience with using *P. antipodarum* for testing EDCs than the other two species, and it is recommended that optimisation and validation of the standard operating procedure (SOP) for this PLC test could begin immediately. A particular issue for optimisation concerns the influence of temperature on the fecundity endpoint, while the focus of validation should be an investigation of the inter-laboratory reproducibility of the test.

VIII. The two FLC procedures with *L. stagnalis* and *C. gigas* require more detailed optimisation before decisions can be made about possible validation. Mollusc reproduction is very sensitive to a wide range of environmental variables, and the relative influence of these factors on the response of these species to EDCs needs to be more firmly established.

IX. The review makes a number of recommendations about data gaps and research needs. In particular, there is a need to establish whether FLC tests with *P. antipodarum* and *L. stagnalis* are consistently more sensitive than equivalent PLC tests, and to discover whether some groups of substances are consistently more potent in *L. stagnalis* than *P. antipodarum*. There is also a need to find out if

sexually dimorphic mollusc species show unique sensitivity to some chemical classes. Finally, to reiterate the points made above, there is a need to optimise the test conditions for all the recommended species.

# Introduction

## 1.1 Background

Since the late 1980s, there has been increasing concern about the discovery that some natural and synthetic substances in the environment are able to interfere with the normal operation of endocrine systems (e.g. Miyamoto and Burger, 2003). These so-called endocrine disrupting chemicals (EDCs) are able to act as hormones (some are, in fact, natural hormones while others mimic hormone action), or block the action of hormones, or interfere with normal hormone transport, metabolism and excretion (Van der Kraak *et al*., 1998). A wide range of impacts on the natural environment has been documented for EDCs, including effects on development, growth and/or reproduction of all major vertebrate groups and some invertebrate phyla (see Matthiessen, 2003 for an overview). Endocrine disrupting effects in humans have been recorded as a consequence of exposure to certain therapeutic agents, and although it remains to be firmly established that EDCs in the environment are also causing such effects, there is circumstantial evidence for this contention (e.g. Sharpe and Skakkebaek, 2003).

It has become apparent that existing OECD Guidelines for the Testing of Chemicals, and other internationally standardised toxicity testing procedures, are not generally of sufficient sensitivity to EDCs for use in environmental hazard and risk assessment. This is because they tend not to expose organisms for parts of their life cycle that are particularly responsive to EDCs (e.g. sexual development; reproduction), or because the endpoints measured (e.g. mortality; short-term development of early life stages) are relatively insensitive to EDCs. This lack of standardised tests for EDCs is becoming a serious problem because several regulations (including the European Union's REACH legislation [CEC, 2007], and the United States Environmental Protection Agency's Endocrine Disruptor Screening Programme set up under the Federal Food, Drug and Cosmetic Act) now require, or shortly will require, consideration of the potential endocrine disrupting properties of chemicals. Under some circumstances, other chemicals regulations (e.g. the European Union Directive on Plant Protection Products [Dir 91/414/EEC and its replacement] and the EU procedure for authorisation of medicinal products for human and veterinary use [EU Regulation EC 726/2004]) may also require long-term reproductive toxicity data on molluscs, although not specifically in relation to endocrine disruption.

Following the publication by OECD of a draft Detailed Review Paper (DRP) on sex-hormone disrupting chemicals (OECD, 2001), the OECD Endocrine Disrupter Testing and Assessment (EDTA) task force has therefore been encouraging the development and validation of new toxicity testing guidelines for potential endocrine disrupters, in line with its Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (Gourmelon and Ahtiainen, 2007; OECD, 2004a). In general terms, Level 3 of the Conceptual Framework calls for the development of *in vivo* assays to provide data on single endocrine mechanisms and effects, Level 4 calls for *in vivo* assays sensitive to multiple endocrine mechanisms and effects, and Level 5 requires apical *in vivo* tests (i.e. those with endpoints relevant for predicting possible impacts on the health of organisms or their populations) sensitive to both endocrine and non-endocrine mechanisms.

By approximately 2009-2010, validated OECD Guidelines on EDC-sensitive toxicity tests are expected to have been agreed for rodents, amphibians, fish, and crustaceans (copepods and mysids). These will include relatively short Level 3 screens for the identification of EDCs (e.g. the 3 week fish screening test, and the

uterotrophic and Hershberger rodent screens), at least one Level 4 developmental test (e.g. the amphibian metamorphosis test), and various Level 5 tests suitable for use in risk assessment, such as partial life cycle tests (e.g. the fish sexual development test), full life cycle tests (e.g. the copepod life cycle test with *Amphiascus tenuiramis*), and possibly some full life cycle or multiple-generation tests with fish and mysids (– see http://www.oecd.org/document/62/0,3343,en_2649_34377_2348606_1_1_1_1,00.html). An outline description of the work on fish tests is given by Hutchinson *et al*. (2003), and that on invertebrates is described by Gourmelon and Ahtiainen (2007). Detailed information can be found in the reports of the OECD Validation Management Group for Ecotoxicity Tests (VMG-eco).

If used in an integrated fashion, this test battery should be sensitive to a wide range of EDCs and non-EDCs, including *inter alia* (anti)estrogens, (anti)androgens, aromatase inhibitors, juvenile hormone mimics, and ecdysone agonists and antagonists. However, although the vertebrates are well-represented in the battery, the only invertebrate representatives to date are the crustaceans. In addition, the OECD Invertebrate Expert Group is beginning to investigate the design and validation of a life-cycle test with an insect *Chironomus riparius* that has aquatic larval stages, but the arthropods remain the only invertebrate phylum currently involved in further guideline development. The importance of this gap in the available suite of ecotoxicity tests is illustrated by the fact that many mollusc populations are known to have been seriously damaged by organotin compounds acting via masculinising endocrine disrupting mechanisms (see more details below), but current internationally standardised tests are not sufficiently sensitive to chemicals with this mode of action in molluscs. The present document will therefore begin to address this shortcoming, partly as a result of the encouragement of the OECD Invertebrate Expert Group at its 2007 meeting in Columbia, South Carolina, which welcomed the potential preparation of a DRP on mollusc testing. Germany and the UK therefore submitted a Standard Project Submission Form (SPSF) to OECD in January 2008 proposing the preparation of such a DRP, and OECD's Working Group of National Coordinators of the Test Guidelines Programme (WNT) approved the SPSF at its meeting in April 2008.

### 1.2 Purpose of the DRP

The purpose of this DRP is primarily to consider whether, and to what extent, molluscs are sensitive to endocrine disruption, to set out what is known about the mechanisms of action of EDCs in molluscs, to describe EDC-sensitive toxicity testing methods with molluscs which might be developed into OECD Guidelines, and to make recommendations about the research and validation activities which might be needed to achieve this objective. It is expected that the review will focus primarily on partial- and full-lifecycle tests which should in principle be sensitive to both EDCs and to chemicals with other modes of reproductive and developmental action.

Why should OECD be concerned about endocrine disruption in non-arthropod invertebrates in general and molluscs in particular? Over 95% of known species are invertebrates, consisting of more than 30 phyla. It will therefore be apparent that the OECD test guideline developments for EDCs described in section 1.1, covering vertebrates and arthropods alone, will not provide representative coverage of much of the animal kingdom. The historical reasons for this are partly the natural incentive to use vertebrates in chemical testing as surrogates for human beings, partly because the arthropods are major pests in agriculture and public health (and hence more familiar to science than other invertebrates), and partly because our knowledge of non-arthropod invertebrate endocrinology is comparatively weak. Nevertheless, some non-arthropod invertebrates are ecologically crucial organisms (e.g. many of the molluscs), whose health and diversity are vital to the biosphere and to the human economy. It is therefore considered important to develop a more balanced suite of tests for endocrine disrupters, some of which substances will inevitably present little or no risk to vertebrates or arthropods.

An illustration of this gap in the suite of available guidelines is the well-known case of the tributyltin-based antifouling paints which have caused masculinisation of at least 150 prosobranch mollusc species worldwide, have decimated many mollusc populations, have severely damaged the oyster-growing industry, and have caused severe losses of invertebrate biodiversity in shallow coastal waters (Matthiessen

and Gibbs, 1998; Matthiessen *et al*., 1999; Rees *et al.*, 1999, 2001; Waldock *et al*., 1999). These impacts have included relatively mild effects on crustaceans, but no known effects on vertebrates. Most of the effects on molluscs were directly or indirectly caused by a type of endocrine disruption, the effects of which, although not yet entirely understood with respect to their mode of action (see section 0 below), would almost certainly not be detected or adequately quantified by any of the new guidelines being developed by OECD. There will no doubt be other examples of EDCs in invertebrates with unique potency for non-arthropods, thus justifying some investigation of the possibilities for new testing procedures.

Another motivation for the development of additional EDC testing procedures with invertebrates is the ethical concern about the use of vertebrates in guidelines developed to date. It seems unlikely that vertebrate-based testing could be totally, if at all, dispensed with in the near future but there may be some scope for using invertebrates to screen for substances with vertebrate sex steroid activity. This approach would help to minimise the numbers of vertebrates sacrificed in chemicals testing. There may also be scope for using cell culture systems to partially replace vertebrate *in vivo* testing. The steroid pattern of cell signalling seems to have been highly conserved in evolutionary history (see section 0, and three major invertebrate phyla that may use steroids for this purpose are the echinoderms, the molluscs and the tunicates (Oehlmann and Schulte-Oehlmann, 2003a). Steroids are also present in some crustaceans, but their functional role and effects in this group are questionable. Their role in molluscs is also questionable, although they may have a function in at least the prosobranch gastropod molluscs (LeBlanc *et al*., 1999; Matthiessen *et al*., 1999). As will be described below (section 0), sex steroids in molluscs appear to be chemically, but possibly not mechanistically or functionally, identical with those in vertebrates, but that would not necessarily preclude molluscs being used in apical tests for EDCs with sex steroidal activity. However, it is recognised that reliable mollusc-based screens for steroidal activity could only be implemented when the role and modes of action of steroids in molluscs are better understood.

There are several reasons why choosing molluscs (rather than echinoderms or tunicates) for further guideline development may prove useful. As well as the endocrinological parallels with vertebrates (which also exist in echinoderms to an even greater extent), it is worth noting that molluscs are second only to the insects as the invertebrate group with the most species (>130,000). Mollusc-based tests may therefore be more amenable to extrapolation in risk assessment programmes than ones based on less numerically important groups. Furthermore, molluscs are of immense benefit to the human economy as sources of naturally-occurring and aquaculture-derived food, so it is important to understand their reactions to synthetic chemicals.

Molluscs are also very diverse in their body patterns and life styles, as will be apparent from consideration of the seven mollusc classes, viz: bivalves; cephalopods, polyplacophorans (chitons), scaphopods (tusk shells), gastropods, and finally the small groups of worm-like aplacophores and monoplacophores. The gastropods are particularly diverse, with 110,000 species, being divided into the archaeogastropods (limpets and topshells etc.), mesogastropods (periwinkles etc.), neogastropods (whelks etc.), tectibranchs (sea slugs), sacoglossids (sea slugs), pleurobranchomorphs (sea slugs), nudibranchs (sea slugs), and pulmonates. This diversity means that molluscs are found in a wide variety of habitats, which is a useful feature when extrapolating test data in environmental risk assessments. On the other hand, a drawback in the present context is that their endocrine systems also vary significantly (see section 0), which makes extrapolation between mollusc classes potentially difficult.

Molluscs are found in all surface waters, but they have radiated most in the sea, and only gastropods and bivalves are found in freshwater. However, freshwater snails may represent up to 20-60 % of the total abundance and biomass of macroinvertebrates in some freshwater ecosystems (Habdija *et al.*, 1995) where they play a major role in the transfer of energy and material across food webs. On the other hand, tunicates and echinoderms are exclusively marine, so it is arguable that they may be less relevant for testing chemicals than gastropods or bivalves which are universally distributed in surface waters. Although aquatic pulmonates usually exhibit lower standing stocks than prosobranchs, their rapid growth and short life cycles frequently result in higher average production rates and shorter turnover times. In addition, the

pulmonate gastropods and slugs are also found in the terrestrial environment. Terrestrial molluscs, however, have been excluded from further consideration in this DRP as there is little demand from regulatory authorities for tests based on this group, and little evidence to date that they have been affected by endocrine disruption.

Another reason for attempting to develop testing guidelines for bivalve or gastropod molluscs is that many are easy to culture and handle in the laboratory, whereas less knowledge about culturing exists for the tunicates and echinoderms. Furthermore, molluscs have been shown to experience at least two types of endocrine disruption in the field (see section 0), and have already been subjected to limited laboratory-based testing for some EDCs (see section 0). This known sensitivity to some EDCs is perhaps the most powerful incentive to develop test procedures with one or both of these molluscan groups. Gourmelon and Ahtiainen (2007), for example, recognise the need for a 'snail' reproduction test guideline, and suggest that a 28 day procedure of this type might be modelled on proposals by Duft *et al*. (2007) for a test based on the freshwater prosobranch (Hydrobiidae) gastropod *Potamopyrgus antipodarum*.

In summary, therefore, the purpose of this Detailed Review Paper is first to consider what is known about the endocrine systems of molluscs and how they can be disrupted by certain chemicals, both in the field and in the laboratory. This is necessary in order to establish firmly the need for test guideline developments in this area, and the scope that such guidelines will ideally need to possess. Secondly, this DRP seeks to consider the various experimental test methods that have been used to investigate the responses of molluscs to chemicals including EDCs, in order to collate and critically evaluate all potentially useful procedures. The third objective is to present as much detail as possible of the most promising available mollusc testing protocols and culture methods, to recommend one or more which appear to have potential for development into OECD Guidelines that can in principle be used to test the long-term toxicity of EDCs and non-EDCs, and to make proposals for any research and method validation that may be necessary. The fourth and final objective is to give practical advice on how the recommended test methods could be implemented, including an evaluation of costs and benefits which can be used by WNT to decide if further OECD work on mollusc testing guidelines is justified.

### 1.3 Objectives of partial- and full mollusc life cycle tests

Consideration of the EDTA Conceptual Framework (OECD, 2004a; Fig. 2-1) shows that there are regulatory requirements for many different types of tests for assessing the properties of EDCs, ranging from diagnostic *in vitro* and *in vivo* screening assays at Levels 2 and 3 respectively to provide information on fundamental properties and modes of action, through to tests which provide some information on processes such as growth, development and reproduction that can be used directly in environmental risk assessments because they are of clear relevance at the population level. At the top of this hierarchy at Level 5 are so-called apical tests which expose organisms for their entire life cycle, or a significant part of it that includes EDC-sensitive processes. It is assumed that single life cycle tests (or multiple life cycle tests in some circumstances) will include all the toxicological processes that could be affected by EDCs (or other chemicals), and they therefore represent the most comprehensive form of testing that is necessary. It should be noted that such tests will not necessarily diagnose the type of EDC to which the test substance belongs, or its mode of action (MOA), but they will provide the best information on toxicity for use in risk assessment. They are therefore equally applicable to all chemicals where high-level toxicity data are required, not just to EDCs. The simpler tests in the Conceptual Framework hierarchy, however, as well as providing mechanistic understanding, can also be used to decide which of the apical tests are the most applicable in given circumstances.

These requirements apply as much to molluscs as to any other group of test organisms. However, in the case of molluscs, we only have a poor understanding of how endocrine disrupters exert their effects (see section 0). Without such knowledge, it is impossible to design robust diagnostic or screening tests. It would be a mistake to argue that there is consequently no point in developing life cycle tests until such screening tests are available, as there is no need for mechanistic understanding in order to evaluate whether a

substance has the potential to damage populations. Whereas life cycle testing may be an expensive strategy when employing organisms with long life cycles, it becomes more practical when testing shorter-lived organisms such as some molluscs.

The primary objective of partial- and full life cycle tests with molluscs at Level 5 is therefore to provide more or less comprehensive information on the apical effects of both EDCs and other chemicals, and not to uncover MOAs, at least not at the present state of knowledge. It is assumed that the results of either partial- or full life cycle tests with molluscs could be used in risk assessment. If sufficient information on a chemical exists then the mollusc results could be used directly in a species sensitivity distribution approach (see Staples *et al*., 2008; Posthuma *et al*., 2002; Selck *et al*., 2002). If insufficient information exists concerning the responses of other species to the chemical then it may be necessary to apply assessment factors or to use other procedures for extrapolating from the laboratory to the natural environment.

Primary endpoints in life cycle tests with molluscs might include some or all of the following:

- Time to spawning
- Fecundity
- Fertilisation success
- Time to hatch
- Hatching success
- Juvenile mortality, growth and development
- Sex ratio
- Gonadal histopathology[1]

As greater understanding of molluscan endocrinology and the responses of molluscs to EDCs is gained, it might be considered desirable to add additional endpoints of a more diagnostic character (e.g. biomarkers of key MOAs, including the expression of certain genes, the activity of certain enzymes or the level of certain metabolic substrates), although it would probably be a more efficient strategy to design separate screening and diagnostic tests that would correspond to lower levels in the EDTA Conceptual Framework.

[1] This can include a range of specific endpoints e.g. oocyte size and number. It should be noted that the conduct of histopathology requires specialist skills and can involve an element of subjectivity, although this can generally be overcome by careful attention to methodology. Histopathology may be useful when assessing sex ratios, and may also provide valuable information about modes of action.

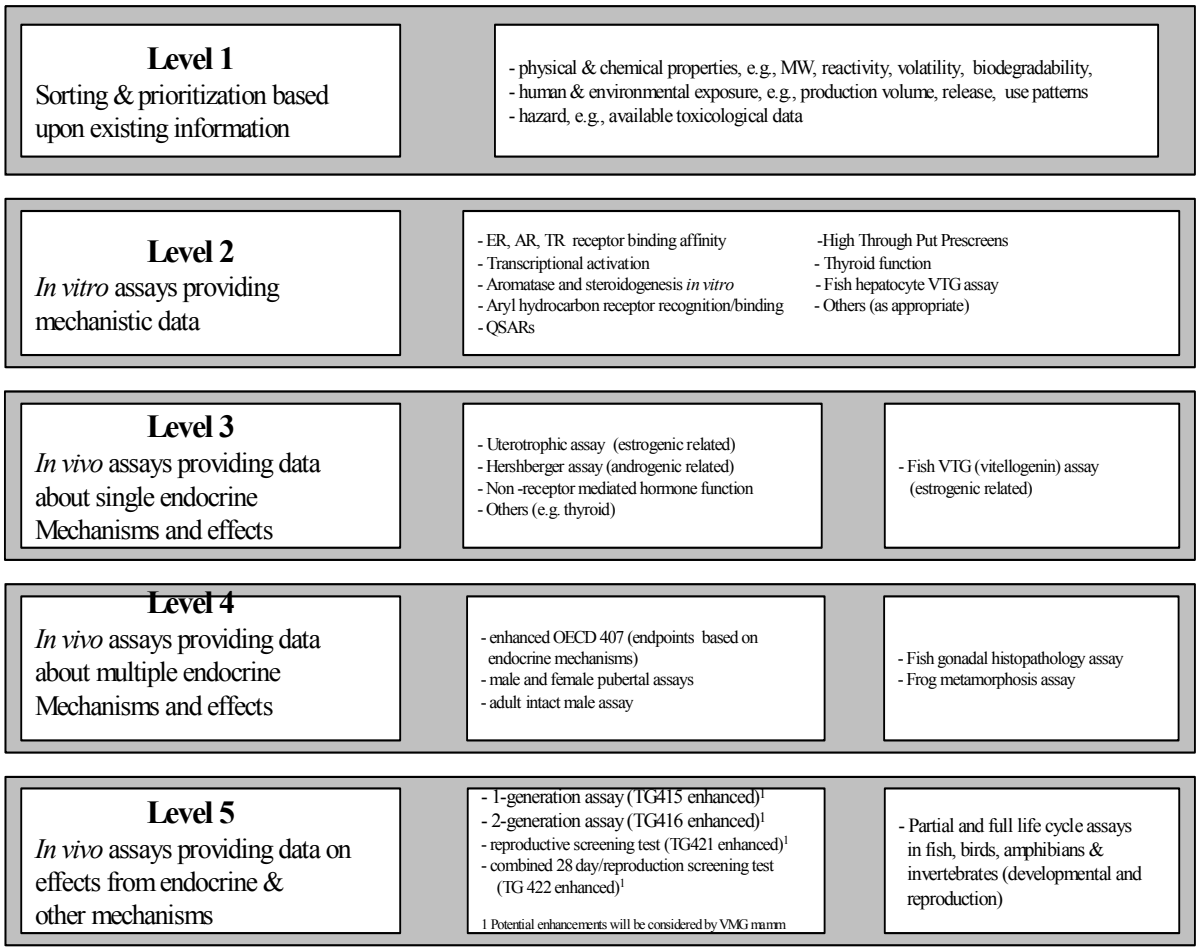


Fig. 2-1. The EDTA Conceptual Framework for the testing and assessment of endocrine disrupting chemicals.

## 1.4 Phylogeny and the divergence of molluscan endocrinology

Evolutionary biologists generally agree that animals diverged into two discrete lineages, protostomes and deuterostomes, during early evolution of the animal kingdom. Accordingly, significant divergences in endocrine strategies would be expected between deuterostomes (the vertebrates and echinoderms without bryozoans) and protostomes (most invertebrate groups including molluscs). Furthermore, the high degree of evolutionary divergence among the individual invertebrate phyla would also allow for significant divergence in endocrine strategies utilised by these phyla (Hoffmann and Porchet, 1984; Dorn, 2000).

This bifurcation seems to demarcate a major divergence in endocrine strategy. Significant evidence exists to indicate that invertebrate deuterostomes use vertebrate-type sex steroids (androgens, estrogens, progestogens) as terminal hormones to regulate reproduction along extended neuroendocrine cascades. On the other hand, available data suggest that protostomes make limited use of vertebrate-type sex steroids for reproductive maturation and function. Most lower protostomes use neuropeptides to regulate these processes; arthropods and particularly insects and crustaceans use a neuroendocrine cascade with the ecdysteroids and terpenoids as terminal hormones (Hoffmann and Porchet, 1984; Dorn, 2000). Ecdysteroids are apparently not part of the deuterostome arsenal of hormones and the deuterostomes make use of terpenoids (i.e. retinoic acid) primarily in patterning early development. Thus, the argument could be made that the endocrinology of deuterostome invertebrates, represented by the majority of echinoderm classes such as sea stars and sea urchins, is more homologous to that of the vertebrates in comparison with the protostome invertebrates (de Fur et al., 1999; Hines et al., 1992). However, there is also evidence for the use of vertebrate-type steroids to control growth, development and reproduction in a number of protostome groups (Lafont, 2000; Lafont and Mathieu, 2007; Pinder and Pottinger, 1999).

Hormonal regulation of biological functions is common to both vertebrate and invertebrate animals but portraying the evolution of hormonal systems is extremely difficult as there are no “fossil hormones” and all conclusions have to be drawn from living organisms. Whereas the evolution of vertebrate organ systems (including the endocrinium) is documented more or less comprehensively, only a few authors have directed their attention to invertebrates and especially molluscs. Therefore most of the information given below refers to the textbook of Kuhlmann and Straub (1985) and the reviews of Hartenstein (2006) and Köhler et al. (2007).

During development of bilaterian organisms, endocrine glands form out of all three germ layers (endoderm, mesoderm, ectoderm). Glands of ectodermal and endodermal origin produce peptide and amine hormones whereas lipid based molecules stem from mesodermal tissue. It is hypothesized that defined epithelial cells embedded in the epidermis and intestinal lining are able to perceive chemical, physical or environmental stimuli which activate metabolite secretion resulting in adaptive responses in other tissues (Hartenstein, 2006).

During the course of evolution, early endocrine cells experienced specializations and separated from the epidermis, neuroectoderm and intestine. Also during evolution, some of these secreting cells migrated to various locations in the body to form independent and true endocrine glands which have only been identified in arthropods and vertebrates. Chemical mediators or hormones exert their regulatory effects at various target sites within the body. Although, a basic endocrine strategy to regulate biological processes has ancient beginnings, individual components intrinsic to the endocrine system have undergone

significant evolutionary divergence resulting in distinct differences in the endocrine systems of various taxa (Hartenstein, 2006; Janer and Porte, 2007; Köhler et al., 2007; Lafont and Mathieu, 2007). Through evolution, invertebrate species in particular have experienced a huge diversity of life histories and have hence adopted a multitude of unique approaches to growth, development and reproduction. These approaches include processes of metamorphosis, diapause and pupation which are life history traits not evident in vertebrates, except metamorphosis in fish and amphibians. Thus, during evolution the neuroendocrine regulation of these processes most likely centred around the uses to which invertebrate hormones were put and was therefore considerably more diverse than that found in the vertebrates (de Fur et al., 1999; Oehlmann and Schulte-Oehlmann, 2002; 2003a).

The archetype mollusc was probably provided with neurosecretory cells interspersed among central and peripheral neurons as well as intestinal cells. By further specialization of the latter, performance and complexity of the molluscan neuroendocrine system was enhanced. In the central nervous system, neurosecretory cells grouped into "nuclei" (Hartenstein, 2006; comp. Figure 3-1). Neurites innervated defined sections of the neuropile and neurosecretory peripheral axons associated with glial sheath, covering the nervous system with blood vessels in several molluscs (Geraerts et al., 1988; Joosse, 1988).

Generally the endocrine system of animals may range from simple neurosecretory sites (potentially involving one or more centres of the nervous system) to complex networks of widely scattered ductless glands that synthesize and release chemical mediators into the circulatory system. Systems of the neurosecretory type may also store and release hormones in neurohaemal organs consisting of neurons directly contacting the circulation system via their endings (e.g. in the molluscs *Helix aspersa, Octopus vulgaris,* and *Eledone cirrhosa*). It is believed that the "unified whole" of gland cell, hormone and target cell did not evolve at the same time, but developed over the course of long evolutionary periods on several occasions (Kuhlmann and Straub, 1985). During this process, two substance groups attained importance as hormones – peptides and steroids. Most likely molecules of the steroid type transferred chemical information from one cell to another. This is indicated by the ability of steroid hormones to permeate phospholipid bilayers and when bound to receptors to directly affect genetically mediated biosynthesis processes.

According to Kuhlmann and Straub (1985) the presence of numerous vertebrate-type hormones in invertebrates can be explained by evolutionary processes of cell metabolism where secretion of these molecules in the first instance was of no endocrine significance, but following the co-evolution of substance-specific receptors some, but not all, of them became functional hormones. Baker (2005) in contrast favours an ancestral nuclear receptor with low ligand selectivity which in co-evolution developed towards a receptor with highly selective affinity for ligand binding, whereas Thornton (2003) argues for the probable appearance of an ancestral ligand-activated receptor. However, as a result, several substances may have survived evolution without retaining a function in recent organisms, while similar molecules in different groups may have different functions or bind species-specifically to different receptors. Receptors may have been structurally conserved but may equally well have occurred convergently several times in the course of evolution. As reported by Köhler et al. (2007), until 2002 sex steroid receptors had only been identified in deuterostomes and it was presumed that these receptors first appeared in a deuterostome archetype. However, Thornton et al. (2003) succeeded in isolating an estrogen receptor (ER) ortholog with close homology to the vertebrate ER genes in the opisthobranch snail *Aplysia californica*. On the other hand, this study revealed the receptor to be a constitutive transcriptional activator, not binding estradiol and independent of hormone regulation. The same phenomenon has been shown for the ER ortholog identified in the cephalopod *Octopus vulgaris* (Keay et al., 2006), the oyster *Crassostrea gigas* (Matsumoto et al., 2007) and the prosobranch snails *Thais clavigera*, *Nucella lapillus* and *Marisa cornuarietis* (Kajiwara et al., 2006; Castro et al., 2007; Bannister et al., 2007).

### 1.5 (Neuro-)endocrine control in molluscs

Unfortunately, there is no source to which one can turn for a comprehensive and authoritative description of molluscan endocrinology, because the field can still be characterised primarily as work in progress, so this section attempts to give a very brief review on the subject.

In vertebrates the "neuroendocrine system" is considered a combination of the endocrine and nervous system in which hormones and electrical impulses serve as messengers to regulate physiological actions in organisms. Whereas the interactions between the nervous and endocrine systems in vertebrates are well known and described, knowledge of function, regulation and especially orchestration of both systems in molluscs is fragmentary.

Generally, the body's signalling system is mediated by local chemical messengers: neurotransmitters, neuropeptides, hormones and pheromones. Histamine is an example of a local messenger or tissue hormone which does not accumulate in the blood and has been identified e.g. in the nervous system of the opisthobranch *Aplysia californica* and the bivalves *Macoma baltica* and *Mytilus edulis* (Karhunen and Panula, 2005; McCaman and Weinreich, 1985; Ungar et al., 1937). Neurotransmitters like acetylcholine are produced by nerve cells and released at their axon terminations to act short-range on adjacent target cells. Early papers by Prosser (1940) and Pilgrim (1954) described action and nervous inhibition of regulator neurons by acetylcholine in the heart of bivalves (*Venus mercenaria, Gryphaea angulata, Mytilus galloprovincialis).* Panchin et al. (2004) applied acetylcholine antagonists to modify the locomotory pattern of the pteropod mollusc *Clione limacina*.

As described by several authors (Geraerts et al., 1988; LeBlanc et al., 1999; Nassel, 1996), the molluscan endocrine system appears to revolve primarily around neurosecretory centres in the cerebral, pleural, pedal and abdominal ganglia which all produce neuropeptides with hormonal action. The primary secretory structures in invertebrates are often neuronal in origin, and termed neurosecretory organs or tissues. As in vertebrates they constitute endocrine active structures associated with the central nervous system (cerebral ganglion) and might be convergent or even analogous to the pineal and/or pituitary glands. The (neuro-)endocrine system of pulmonate snails is by far the best-investigated. Multitudinous hormonally active regions have been identified as integral parts of their nervous system and Pinder et al. (1999) provide a detailed overview of this topic which is briefly summarized below. So-called dorsal bodies can be found on the lateral surface of the cerebral ganglia of pulmonates. Dorsal body cells are known to produce the dorsal body hormone (DBH) which was originally characterised as a female gonadotropic hormone. As it was presumed that the dorsal bodies are of mesodermal origin (Boer et al., 1968) they are additionally considered to synthesize steroid hormones. Furthermore caudo-dorsal cells have been identified to produce the caudo-dorsal peptide hormone (CDCH) that plays an important role in egg-laying. The light green cell system of the cerebral ganglia *inter alia* releases neurohormones mediating growth and molluscan insulin-related peptides. However, embedded in the lateral lobes of the cerebral ganglia are cells producing inhibitory growth factors. The dark green cell system can be found in the pleural ganglia of pulmonates and generates a hormone, structurally analogous with vertebrate thyroid-stimulating releasing hormone (TRH), with diuretic effect. The dark green cells are supported by yellow and yellow-green cells which direct osmotic events by the synthesis of sodium influx stimulating peptide (SIS).

As illustrated by Saleuddin et al. (1994) in pulmonate landsnails, dorsal body cells are dispersed throughout the connective tissue that encloses the nerve cells, similar to the situation in proso- and opisthobranchs where these structures are termed 'juxtaganglionar organs'. In cephalopods the so called optic glands represent the central nervous system (CNS)-associated endocrine areas (comp. Figure 3-2).

Neuropeptides or neurohormones are released to body fluids and transported to nonadjacent target cells where they unfold their potential and for which some substantial information is available (Lubet and Mathieu, 1990).

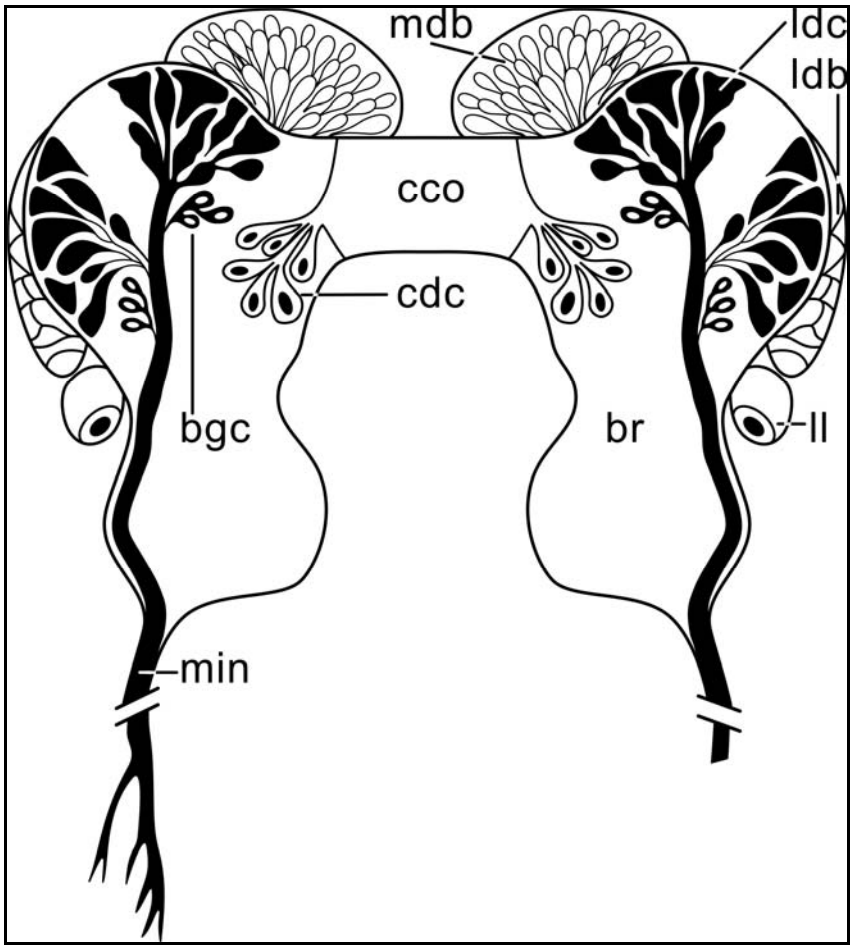


Figure 0-1: Overview of elements of the pulmonate neuroendocrine system modified after Hartenstein (2006). Schematic dorsal view of gastropod cerebral ganglion. Central nervous system is coloured white. Multiple clusters of central neurosecretory cells are present in the "brain" (bgc, bag cells; cdc, caudo-dorsal cells; and ldc, latero-dorsal cells). In some cases, neurohaemal release sites have been identified (cco, commissural neurohaemal organ; and min, median lip nerve). The medio-dorsal body (mdb), latero-dorsal body (ldb), and lateral lobe (ll) form endocrine structures closely associated with the brain and targeted by neurosecretory cells.

Most work has been restricted to relatively few species of opisthobranch and pulmonate gastropods, particularly *Aplysia* and *Lymnaea* while there is some information on the bivalve *Mytilus* (de Lange and van Minnen, 1998; Lagadic et al., 2007; Nassel, 1996; Norekian and Satterlie, 1997; Santama et al., 1996). Lagadic et al. (2007), who are the prime source for this paragraph, describe the considerable amount of information about the neuroendocrine control of reproduction in the hermaphrodite *Lymnaea stagnalis*. Most of the eleven groups of hormones identified in *L. stagnalis* to date are indeed neuropeptides. However, the chemical nature of the DBC hormone, which controls the female accessory sexual organs, vitellogenesis, oocyte maturation and galactogen synthesis in the albumen gland, is still unresolved. Experiments suggest that dorsal body cells either synthesize a protein hormone and a steroid hormone, or a steroid hormone together with its binding protein. Peptides secreted by the caudodorsal cells and other parts of the central nervous system regulate the onset and control of egg-laying behaviour, and include the so-called ovulation hormone (CDCH-1) which causes the release of mature oocytes by the ovotestis, and

FMRFamide. The albumen gland in turn produces epidermal growth factor (L-EGF) and trypsin inhibitor (LTI), both involved in embryonic development. The lateral lobes exert global control of female reproductive activity, and are associated with a peptide similar to gonadotropin-releasing hormone (GnRH). The endocrine functions of the ovotestis are not clear, but the associated Sertoli cells are important in this regard, and may control spermatogenesis and spermiation. Male copulatory behaviour is under the control of peptidergic neurons – APGWamide modulates the muscles involved in everting the preputium, and antagonises the effects of another neuropeptide (conopressin) that causes the vas deferens to contract and ejaculate semen.

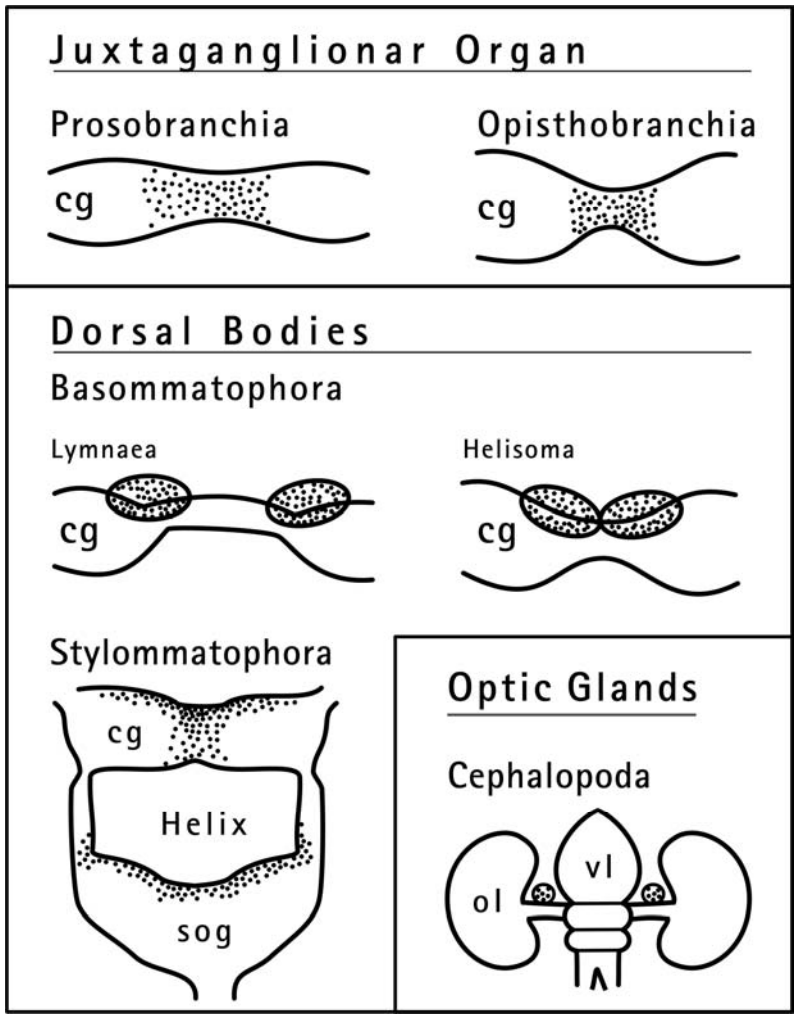


Figure 0-2: CNS-associated endocrine structures (dotted areas) in major molluscan groups (modified after Saleuddin et al. 1994). cg, cerebral ganglion; ol, optic lobe; sog, subesophageal ganglia: vl, ventral lobe.

The occurrence of gonadotropin-releasing hormone is not restricted to vertebrates (for details see review of Tsai, 2006). Several studies have substantiated the presence of this molecule in many invertebrate groups including gastropods (*Aplysia californica, Helisoma trivolis, Lymnaea stagnalis*), bivalves (*Crassostrea gigas, Mytilus edulis*) and cephalopods (*Octopus vulgaris*). Hatcher and Sweedler (2007) grant special importance to the role of GnRH neurones when speculating on the similarities of neuroendocrine networks in mammals and the reproductive bag cell neuroendocrine system of the sea slug *Aplysia californica*. Bag cells are known to regulate the reproductive effort in *Aplysia* through release of peptides derived from the

egg-laying prohormone (proELH) into the neurohaemal region of the nervous system (abdominal, pleural and cerebral ganglia), similar to GnRH neurones when activating the hypothalamic pituitary axis. It has been shown that the bag cell network operates via descending action potentials which transmit from cerebral and pleural ganglia to abdominal bag cell clusters. Additionally Antkowiak and Chase (2003) demonstrated ovotestis innervation with sensory function in the pulmonate snail *Helix aspersa* and concluded that sensory endings in the gonad correspond to oocyte maturation and neural CNS feedback.

With the exception of a number of French studies from the 1970s, focussing on the endocrine control of sex change in the protandric hermaphrodite *Crepidula fornicata* (e.g. Bardon et al., 1971; Le Gall et al., 1983; Le Gall and Streiff, 1978; Ny and Le Gall, 1976) little basic research has been done with prosobranch snails. The experiments with *Crepidula* investigated the role of "neuroendocrine factors", presumably neuropeptides, on penis formation and regression during sexual differentiation and the sex change from males to females. These studies provided evidence that the pedal ganglia in the prosobranch central nervous system produce a "penis morphogenesis factor" in both sexes of snails or in the male and female phases of consecutive hermaphrodites. A "penis regression" factor is produced exclusively by the pleural ganglia of females or hermaphrodites in the female phase. Based on a number of convincing experiments it was concluded that a penis is formed under "penis morphogenesis factor only" conditions, i.e. in males, the male phase of hermaphrodites, or if production of the "penis regression factor" is inhibited. The authors did not characterise these neuroendocrine factors nor did they investigate the potential role of steroids in the process of penis formation or suppression. However, according to Saleuddin et al. (1994) juxtaganglionar organs in proso- and opisthobranchs (as well as the optic glands of cephalopods and dorsal bodies of pulmonates) are considered to produce female gonadotropic hormones which are involved in the neuroendocrine control of reproduction (e.g. egg maturation, albumen and vitellogenin production and size of accessory sex organs).

The cardio-acceleratory peptide FMRFamide was first characterized in the clam *Macrocallista nimbosa* and the head ganglia of *Aplysia brasiliana* (Lehmann et al., 1984; Price and Greenberg, 1977). The FMRFamide family is now the best known, characterised and most widespread of all invertebrate neuroendocrine hormones. Its activities in molluscs are manifest in a range of physiological processes of which the regulation of heartbeat is the best characterised (Greenberg and Price, 1983; Favrel et al., 1998; Nassel, 1996; Santama et al., 1996; Suzuki et al., 1997; Tensen et al., 1998).

Molluscan insulin-like peptides (MIPs) also produced by central nervous system cells are amongst the best-characterised peptides. There are at least six members of this structurally related family (Smit et al., 1996) that bear striking resemblance to the vertebrate insulins and insulin-like growth factors. They have established roles in growth, development and metabolism (Pertseva et al., 1996).

In molluscs, shell growth seems to be under neuroendocrine control, as shown for *Lymnaea stagnalis* and *Helisoma* sp. where the growth hormone-producing light green cells of the cerebral ganglia possess a hormone-dependent calcium-binding protein (CaBP) (Dogterom and Doderer, 2006; Saleuddin and Kunigelis, 1984). In addition, Ottaviani and Franceschi, (1996) and Ottaviani et al. (1991, 1992a,b, 1993, 1994) demonstrated in several studies the presence of the catecholamines adrenaline, noradrenaline and dopamine in the central nervous system (CNS) of prosobranch and pulmonate snails (*Helicella virgata, Planorbarius corneus, Viviparus ater*). These authors furthermore observed that molluscan haemocytes were equipped with the enzymes tyrosine hydroxylase and dopamine *ß*-hydroxylase which are essential for the biosynthesis of biogenic amines (Ottaviani et al., 1993).

The best studied endocrine-related communication system among invertebrates is the neuropeptide signalling mechanism although non-peptide hormones, including the two key ones, ecdysone and juvenile hormone (JH) are important in a number of phyla (Novales et al., 1973). Ecdysteroids and JHs have been reported in a few molluscan species but remain unconfirmed in the majority. Data are inadequate to confirm that ecdysteroids play an important role in molluscan physiology (Lafont and Mathieu, 2007). In arthropods, ecdysone was first recognized for its role in molting and JH for its role in metamorphosis of insects. However, these hormones are now known to regulate a variety of aspects relating to development,

growth, metabolism, and reproduction.

Table 3-1: Steroid hormones which have been identified analytically in various mollusc taxa (modified after Janer and Porte, 2007).

| Species | Hormone |
|---|---|
| **Gastropoda/Prosobranchia** | |
| | |
| *Marisa cornuarietis* | Testosterone, estradiol |
| *Bolinus brandaris* | Testosterone, estradiol |
| **Gastropoda/Pulmonata** | |
| | |
| | |
| | |
| *Helix aspersa* | Dihydroepiandrosterone, androsterone, androstenedione, testosterone, 5α-dihydrotestosterone, 3α-androstanediol, estrone, estradiol, estriol, progesterone |
| *Arion ater rufus* | 11-Ketotestosterone, testosterone, 17α-hydroxyprogesterone, estrone, estradiol |
| **Bivalvia** | |
| *Crassostrea gigas* | Estradiol, estrone |
| *Mya arenaria* | Testosterone, estradiol |
| *Mytilus edulis* | Testosterone, androstenedione, progesterone, estradiol, estrone |
| *Mytilus galloprovincialis* | Testosterone, estradiol, estrone |
| *Patinopecten yessoensis* | Estrone, estradiol |
| *Ruditapes decussata* | Testosterone, estradiol |
| **Cephalopoda** | |
| *Octopus vulgaris* | Testosterone, progesterone, estradiol |

The endocrinology of the various classes of molluscs and even of major groups of gastropods – prosobranchs, opisthobranchs, and pulmonates – varies greatly, reflecting extreme differences in morphology and life histories (Lafont and Mathieu, 2007; Lagadic et al., 2007). This can be exemplified by the vertebrate-type steroids, which occur in prosobranchs and probably have a functional role (LeBlanc *et al*., 1999; Matthiessen *et al*., 1999). In the 'older literature' a number of reports of steroid biosynthesis and metabolism in prosobranch snails can be found (e.g. Rohlack, 1959; Lehoux and Williams, 1971). Vertebrate-like steroids, however, have been measured more recently in several species of molluscs (comp. Table 3-1) and biosynthesis studies following the pathways taken by labelled precursors suggest the ability

to synthesise a range of androgens and estrogens (Lafont and Mathieu, 2007; Ronis and Mason, 1996; Wootton et al., 1995). In numerous publications the biosynthesis of vertebrate-type steroids has been demonstrated for molluscs (e.g. Bardon et al., 1971; Bose et al., 1997; D'Aniello et al., 1996; Janer and Porte, 2007; de Jong-Brink et al. 1981; de Longcamp et al., 1974; Hines et al., 1996; Krusch et al., 1979; Le Curieux-Belfond et al., 2001; Lupo di Prisco and Dessi'Fulgheri, 1975; Lupo di Prisco et al., 1973; Ottaviani and Franceschi, 1996; Siah et al., 2002).

Several studies have also implicated steroidal androgens in sex differentiation of molluscs. For example, testosterone administration to castrated male slugs (*Euhadra prelionphala*) stimulates the production of a head wart, a male secondary sex characteristic in this species (Takeda, 1980). Similarly, administration of testosterone to female gastropods causes these organisms to develop imposex with extensive penis and vas deferens formation (Bettin et al. 1996; Spooner et al., 1991). In contrast, there is no clear indication that opisthobranchs and pulmonates (with the possible exception of the above-mentioned *Euhadra* species) use steroidal sex hormones although the presence of testosterone, estradiol and progesterone is well-established in these gastropods, and in a range of cephalopods and bivalves.

Although the functional role of sex steroids in the entire phylum of molluscs has been questioned (Thornton, 2003; Thornton et al., 2003; Nishikawa et al., 2004), it is evident that such steroids exert marked effects when administered to a wide range of molluscan species, as is evidenced, for example, by induction of male-to-female sex reversal in E2-injected oysters, mussels and other bivalves (e.g. Mori, 1969, Mori et al., 1969, Wang and Croll 2003), the E2-mediated induction of a type of vitellin in bivalves (Osada et al., 2003), and the fact that steroid titres and gonad development in bivalves such as clams and mussels are often correlated (Lafont and Mathieu, 2007). This is furthermore exemplified by the fact that natural and synthetic steroids such as E2, EE2, T and MT have been applied at concentrations in the mg/L range to produce pure female or male offspring in mussel and oyster mariculture (Wang, 2000). There is no reason to suppose that exclusively exogenous steroids alter growth, development and reproduction in molluscs while endogenous steroids are without any effect. However, a complete steroid biosynthetic and receptor-binding scheme has not been described for any mollusc species, and the tissues involved in steroidogenesis have not often been identified, although the basic metabolic picture is very similar to that in mammals (Figure 3-3). The primary gap in our knowledge is not so much the presence and synthesis of steroids in many molluscs, which is undisputedly similar to that in vertebrates, but the precise way in which these steroids exert endocrine control in this phylum.

Overall these findings are suggestive for the existence of functional sex steroid receptors in bivalves, gastropods and cephalopods, although known mollusc ERs do not bind 17ß-estradiol and an AR has not yet been cloned for a mollusc species. Nevertheless, Köhler et al. (2007) recommend consideration of the potential limitations and significance of the different methodological approaches applied. ER-like proteins detected by immunochemical methods have been reported for the bivalves *Mytilus galloprovincialis, Pecten maximus, Pecten yessoensis, Mytilus edulis* and the cephalopod *Octopus vulgaris* (Canesi et al., 2004; Di Cosmo et al., 2002; Dorange and Le Pennec, 1989; Osada et al., 2003; Stefano et al., 2003; Won et al., 2005). However, the ability of antibodies to react with similar antigenic sites on different proteins (antibody cross reactivity) must be seen as a disadvantage of immunochemical assays and clearly reduces the usefulness of these methods to demonstrate steroid receptor presence unless the antibodies have been directed specifically at the mollusc receptors themselves.

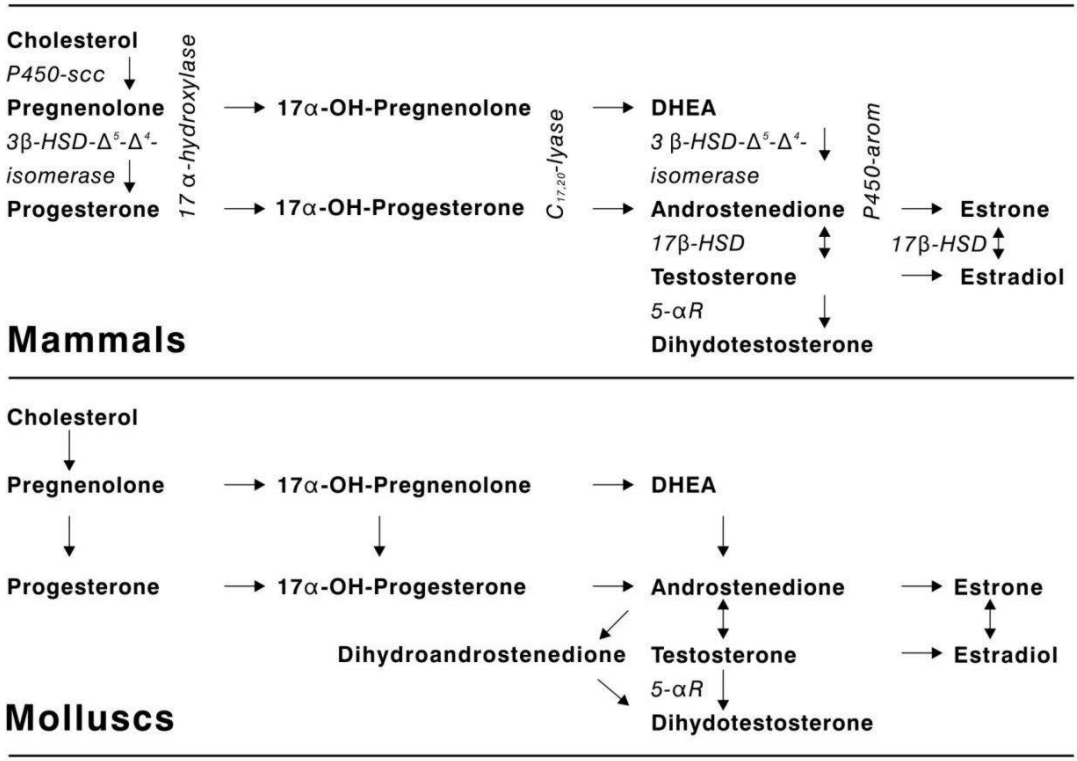


Figure 3-3: The similarity of steroidogenic pathways in mammals and molluscs (modified after Janer and Porte, 2007).

Experiments focussing on the pharmacological elimination or blocking of sex steroid receptors have suggested that receptor-mediated sex steroid signalling occurs in the gastropod *Nucella lapillus*, and the scallop *Placopecten magellanicus* (Bettin et al., 1996; Santos et al., 2005; Wang and Croll, 2003). Although these studies indicate the presence of sex steroid receptors they do not offer evidence about molecular receptor structures. Ligand-binding studies (e.g. by radiolabelling assays) revealed sex steroid binding in tissue homogenates of the mussel *Elliptio complanata* and the prosobranch snail *Marisa cornuarietis* (Gagné et al., 2001; Oehlmann et al., 2006) but, as Köhler et al. (2007) point out, the presence of binding sites does not necessarily indicate the presence of a receptor.

Köhler et al. (2007) have emphasised that although findings of an ER protein in mollusc tissues postulate a corresponding encoding gene, the reports about such a high sequence homology between a mollusc and a mammalian species have to be handled with care since, even within the mammalian class, ER gene sequences obtained from different species are far from being identical. Therefore homologues from molluscs may have different functions than the complementary ER in vertebrates, or steroid signalling in invertebrates may be mediated via pathways unknown in vertebrates or not involving vertebrate-like ERs. Nevertheless, efforts have been made to isolate estrogen receptor-like orthologs in several molluscan species and researchers have succeeded in demonstrating their existence in the gastropods *Aplysia californica, Thais clavigera, Marisa cornuarietis* and the cephalopod *Octopus vulgaris* (Bannister et al., 2007; Kajiwara et al. 2006; Keay et al., 2006; Thornton et al., 2003). The crucial factor is that, although many molluscs have steroid receptors that are very similar to those in vertebrates, they do not seem capable of mediating steroid action. This is not the place for a detailed treatment of this issue, but Janer and Porte (2007) speculated that steroid hormones may act via cell-surface receptors in molluscs, in association with steroid binding proteins in the blood i.e. they may operate via signal transduction pathways similar to those involving peptide hormones.

## 1.6 Modes of action of endocrine disrupting chemicals (EDCs) in molluscs

Even determining whether a single chemical, especially at sublethal concentrations, can impact endocrine-driven functions in a feral population of molluscs is difficult and may require a prolonged and in-depth understanding of their life history, morphology and the influence of local environmental conditions. In general, responses of invertebrate endocrine systems to endocrine disrupters have not been well studied compared to those in mammals or other vertebrates (Baldwin et al., 1995; Colborn et al., 1993; Fox, 1992). Molluscs have complicated life histories, display various forms of hermaphroditism (Novales et al., 1973) and in some cases display poorly resolved sexual dimorphism (Sellmer, 1967). Their reproductive cycle can be highly complex (Sastry, 1968, 1970) and controlled by many environmental stimuli including light intensity, temperature, desiccation and diet (Ansell and Trevallion, 1967; Copeland and Bechtel, 1974; Largen, 1967). In the past, studies of hormonally active substances, whether in molluscs or other clades mainly focussed on sex steroids and the impairment of reproduction (e.g. Bettin et al., 1996; Duft et al., 2003b; Jobling et al., 1995, 1996; Oberdörster and McClellan-Green, 2002; Oehlmann et al., 2006). Given the complexity of endocrine systems, there are many ways in which endocrine disrupting chemicals can affect the body's signalling system and this makes unravelling the mechanisms of action of these molecules difficult. Indeed, there might be a number of endocrine tissues (e.g. nervous system, kidney, midgut gland) affected by EDCs, producing hormones other than sex steroids (e.g. neuroendocrine hormones like MIP, growth hormones, FMRFamide, catecholamines, ACTH-like steroids etc.), but such effects are hardly known.

When defining how endocrine disrupters work in molluscs the crux of the matter is that although many more-or-less apical effects of these substances are known, basic information about their modes of action on the molluscan endocrine system is fragmentary. The review of Sanderson (2006) exemplifies that even within the vertebrate clade with its much better characterised endocrine system, MOAs of endocrine active substances are diversified, not understood in detail and often defined by applying the exclusion principle.

Generally, substances possessing hormonal activities are found in diverse groups of man-made and natural chemicals (e.g. pesticides, plasticizers, alkylphenol ethoxylates as industrial surfactants, organotin compounds, PCBs, phytestrogens, natural hormones) and in pharmaceuticals (e.g. ethinylestradiol, methyltestosterone, trenbolone). From a mechanistic point of view, this makes it impossible to assign a unique mode of action to EDCs in general. While some of the substances take effect by binding but not activating the receptor (antagonistic effect) others may mimic biological activity by attaching to the receptor to produce hormone-like action in an agonistic manner. According to Monod et al. (2004), in rainbow trout, imidazole (prochloraz, imazalil) and triazole (epoxiconazole) fungicides significantly enhance oocyte maturation which is normally induced and regulated by gonadotropin release. Actinomycin D (mRNA biosynthesis inhibitor) was able to completely inhibit oocyte maturation induced by the fungicides, suggesting that the gonadotropin-like agonistic effect of these chemicals depends on de novo gene expression. Furthermore, it has been found that the antiandrogenic effects of p,p'-DDE, vinclozolin and metabolites result from their ability to prevent androgen binding to the androgen receptor (AR) (Kelce et al., 1994, 1995) and finally the suppression of androgen target gene expression (Kelce et al., 1994, 1995; Wong et al., 1995). Interestingly, these studies also provided evidence that depending on ligand binding affinity, concentration and presence of competing natural ligands, androgen antagonists can also act as agonists.

Endocrine toxicants may furthermore act via non-genomic MOAs (i.e. non-receptor mediated). Some chemicals are known to bind to hormone transport proteins (CBG, SHBG, TBG, GHBP) or to alter enzyme activities or other metabolic pathways to affect the synthesis of endogenously produced hormones (see also indirect TBT MOAs below). In competitive binding experiments brominated flame retardants, tetrabromobisphenol A, and several organochlorine compounds turned out to bind with great affinity to the thyroid serum transport protein transthyretin but not necessarily to the thyroid receptor (Cheek et al. 1999, Ilonka et al. 2000), which in vivo may well lead to alterations of the endogenous thyroid hormone titre.

As outlined below, the best known chemical causing endocrine disruption in molluscs (TBT) likewise

appears to possess one or more MOAs. Oehlmann et al. (2007) highlighted that even for a single endpoint like TBT-induced imposex development in prosobranch molluscs, several hypotheses about potential modes of action exist. These include vertebrate-type steroid hypotheses (including aromatase inhibition, inhibited testosterone excretion, modulation of free and fatty acid-bound testosterone levels), a neuropeptide hypothesis, and a retinoid X receptor hypothesis. Beyond these possible MOAs, the same substance is known or suspected to produce a range of further, not necessarily endocrine-mediated, effects like shell deformation, reduced spat fall, altered development rates and growth in bivalves, inhibition of calcification in the skeleton of corals, cytotoxic effects like apoptosis and compromised mitochondrial membrane integrity in mammals, fish and tunicates, and damage of the immune and auditory system of marine mammals (e.g. Allemand et al., 1998; Alzieu et al., 1982; Cima and Ballarin, 1999; Coelho et al. 2001; Song et al., 2005; Tiano et al., 2003).

### 1.6.1 Possible TBT MOAs: Vertebrate-type steroid hypothesis (aromatase inhibition)

Spooner et al. (1991) and Stroben et al. (1991) proposed that TBT may induce imposex in prosobranchs by inhibition of cytochrome P450-dependent aromatase (CYP19). These findings in TBT-exposed snails were characterized by a dose- and time-dependent increase of testosterone (T) titres. Furthermore an administration of T (via injection or via water) also induced imposex. The hypothesis was substantiated experimentally by Bettin et al. (1996). The authors confirmed a positive correlation between imposex stages and T levels in wild-caught *Nucella lapillus* and *Nassarius reticulatus*. For both species, a dose- and time-dependent increase of T titres under TBT exposure in the laboratory was reported. The authors showed that imposex development was inhibited in snails under co-exposure to TBT and the AR antagonist CPA, suggesting that T and the AR play key roles in imposex induction. Furthermore, aqueous exposure to a specific CYP19 inhibitor (formestane at 0.3 mg/L) resulted in increased imposex levels.

Lu et al. (2002) developed an enzyme-linked immunosorbent assay (ELISA) for the determination of T levels in reproductive organs of single specimens of the rock shell *Thais clavigera*. The results demonstrated significantly higher T concentrations in imposex-affected females than in snails from uncontaminated sites, although Lu et al. (2001) reported the detection of EE2, a synthetic steroid, in ovaries of the ivory shell *Babylonia japonica*, which suggest that identification of steroids by high resolution GC/MS does not necessarily mean that gastropods synthesise these steroids but may accumulate them in contaminated environments. Santos et al. (2002) sampled normal and imposex affected female *Buccinum undatum* from the open North Sea at three locations, one with low, and two with high-shipping densities. CYP19 activity was significantly higher in normal females collected in the low-shipping density area than in imposex-affected animals from high shipping density areas. Barroso et al. (2005b) analyzed organotin body burdens, imposex and steroid hormone levels (T, T glucuronide, T sulfate conjugates, and E2) in natural populations of *N. reticulatus* from Portugal between 1997 and 1999. T levels in females without imposex were always lower than in females with imposex, and the T/E2 ratio tended to increase with increasing imposex and organotin contamination. Santos et al. (2005) used *N. lapillus* from an almost imposex-free population, and TBT at a concentration of 50 ng as Sn/L as positive control. The same CYP19 inhibitor as in the study of Bettin et al. (1996) was applied at the identical concentration and again induced imposex, whereas CPA blocked the capacity of TBT to induce imposex. The determination of steroid levels in female specimens revealed that TBT induces an elevation of free T but not of the total amount (free+esterified).

### 1.6.2 Possible TBT MOAs: Vertebrate-type steroid hypothesis (inhibited testosterone excretion)

Ronis and Mason (1996) observed an inhibition of sulfation of T in in vivo and in vitro experiments with TBT-exposed periwinkle *Littorina littorea*, resulting in a decreased metabolic elimination of the steroid. TBT-exposed gastropods retained significantly more T from an administered [$^{14}$C]-T dose than unexposed snails. This was explained by a reduced rate of conversion of T to its sulfate conjugates, thus impeding T elimination. The results of Ronis and Mason (1996) have not been confirmed in other studies at

environmentally relevant TBT concentrations. It is also worth mentioning that the authors found a 30–40% inhibition of CYP19 activity in their experiments with *L. littorea*.

### 1.6.3 Possible TBT MOAs: Vertebrate-type steroid hypothesis (modulation of free versus fatty acid-bound testosterone levels)

Gooding and LeBlanc (2001) questioned the general comparability of T metabolism and disposition between snails and vertebrates and characterized T biotransformation in the mudsnail *Ilyanassa obsoleta*. T was not readily eliminated by this species, nor did the authors detect a production of polar T conjugates. Hydroxy metabolites and oxido-reduced derivatives were relatively minor products. T was largely retained as non-polar fatty acid conjugates, and this led to the hypothesis that esterification of T to a fatty acid ester could be a strategy for steroid regulation and could be involved in the development of imposex.

In a follow-up study, Gooding et al. (2003) investigated whether TBT interferes with the esterification of T, resulting in elevated free (unesterified) T levels associated with imposex. Exposure of snails to TBT concentrations at or above 1 ng as Sn/L induced imposex but did not affect total (free+esterified) T levels in snails. However, free T levels increased with increasing exposure to TBT. In TBT-exposed *I. obsoleta* the production of [$^{14}$C]-T-fatty acid esters from administered radio-labelled precursor decreased with increasing TBT concentrations, indicating that TBT may act via a suppression of acyl coenzyme A (CoA) testosterone acyltransferase (ATAT), an enzyme that converts T to the fatty acid ester. In a later study Sternberg and LeBlanc (2006) have also shown that TBT inhibits ATAT activity. Gooding et al. (2003) concluded that the target of TBT may be a co-contributor to the T fatty esterification process, or a factor in the enhanced hydrolysis of the T-fatty acid pool. Gooding and LeBlanc (2004) found in wild snails that T existed predominantly in the free, non-esterified form at the onset and end of the egg laying period while at other times, the majority of T was sequestered as fatty acid esters.

Recently, Janer et al. (2006) investigated sex differences in endogenous levels of esterified steroids in *Marisa cornuarietis*. According to the findings of Gooding and LeBlanc (2001) for *I. obsoleta*, T and E2 were mainly found in the esterified form in the digestive gland/gonad complex, and males had higher levels of esterified steroids than females (4–10-fold). Additionally, the ability of several xenobiotics (TBT, MT, and FEN) to interfere with the esterification of T and E2 was investigated. All three compounds induced imposex but had different effects on steroid metabolism. TBT exposure led to a decrease in both esterified T (60–85%) and E2 (16–53%) in females, but had no effect on males. Exposure to FEN and MT did not alter the levels of esterified steroids in males or in females, although exposed females developed imposex. The decrease in esterified steroids caused by TBT could not be directly linked with a decrease in ATAT activity. Contrary to expectations, ATAT activity was marginally induced in TBT-exposed snails after 50 days (1.3-fold), and significantly induced in males and females exposed to MT for 50 days (1.8- and 1.5-fold, respectively), whereas no effect on ATAT activity was observed after 150 days exposure.

### 1.6.4 Possible TBT MOAs: Neuropeptide hypothesis

Results from the early 1980s demonstrated that TBT inhibits the release of a neuroendocrine factor [Penis Regression Factor (PRF)] from the pleural ganglia, which is responsible for the suppression of penis formation in females, resulting in imposex development (Feral and Le Gall 1982). The authors did not find any effects of TBT on the formation of the Penis Morphogenic Factor (PMF), which is expressed in all prosobranch snails, irrespective of their sex. Although both factors have not been identified, recent results show that administration of the neuropeptide APGWamide can significantly induce imposex in *I. obsoleta* at $10^{-16}$ M subcutaneous injection over 2 weeks (Oberdörster and McClellan-Green 2000). Oberdörster and McClellan-Green (2002) proposed that APGWamide could represent the PMF in this species. Furthermore, the authors found some support for the aromatase inhibition hypothesis because in vitro studies with digestive gland microsomes resulted in a 52% reduction in CYP19 activity in TBT-dosed snails. The authors concluded that a combination of changes in peptide and steroid hormones may be involved in

imposex induction. Further support for the neuropeptide hypothesis was found in analyses of APGWamide levels in TBT- and T-treated mudsnails (Oberdörster et al. 2005). While control males had significantly higher APGWamide levels than control females, all TBT-treated animals (male, female, and imposex) had APGWamide levels similar to control males and significantly higher than control females. In T-treated animals, APGWamide levels were the same as controls and it is likely that T interferes with downstream signalling in imposex induction. These latest results by Oberdörster et al. show that the aromatase inhibition and neuropeptide hypotheses are not necessarily mutually exclusive.

### 1.6.5 Possible TBT MOAs: Retinoid X receptor hypothesis

Nishikawa et al. (2004) and Horiguchi et al. (2007) have shown that organotin compounds interact with the RXR in humans and the rock shell *T. clavigera*. TBT and TPT exhibit a high affinity to the human RXR. The injection of its suspected natural ligand 9-cis retinoic acid (RA) into female snails induced imposex development. An analysis of cloned RXR homologues from *T. clavigera* revealed that the ligand-binding domain of the snail RXR is similar to the vertebrate RXR, displaying comparable affinities to both 9-cis RA and organotin compounds. From their experiments, the authors concluded that the RXR plays an important role in inducing imposex as RXR gene expression was significantly higher in penises of males and imposex-affected females compared to presumptive penis tissue of normal females ($P<0.01$ and $P<0.05$, respectively).

Furthermore, Le Maire et al. (2009) have shown that organotin compounds bind to and activate the human peroxisome proliferator-activated receptor (PPAR, subtype γ) as well as to all three RXR-α–PPAR-α, -γ, -δ heterodimers at environmentally relevant concentrations. However, there is no published evidence for the existence of PPARs in molluscs.

The results from Nishikawa et al. (2004) with the supporting evidence from Horuguchi et al. (2007) clearly point to a potential alternative MoA for imposex induction by organotin compounds, supported by *in vitro* and *in vivo* results. Dmetrichuk et al. (2008) have recently detected all-trans retinoic acid and 9-cis RA in the central nervous system of *Lymnaea stagnalis* by high-performance liquid chromatography/mass spectrometry and have linked their physiological role to neuronal regeneration and axon pathfinding. Carter et al. (2005) have also published an RXR sequence from *L. stagnalis* in Genbank (GenBank accession: AY846875). Beside the fact that Werner and DeLuca (2001) question the role of 9-cis RA as the physiological RXR ligand because the compound could not be identified in rat tissues, it has been shown for the fiddler crab *Uca pugilator* that a simultaneous expression and association of the RXR and the ecdysteroid receptor (EcR) is required to bind to responsive DNA elements (Durica et al. 2002). Other receptors, including the AR, are also reported to form heterodimers with the RXR (Chuang et al. 2005). According to these results, the AR and RXR mutually affect their transcription and it cannot be excluded that RXR activation might produce androgenic but also antiandrogenic effects by this mechanism.

These conflicting results may reflect species-specific differences in imposex induction and its underlying mechanism. A species specificity has also been demonstrated for imposex induction by TPT (see above) and by the remarkable observation that some neogastropods such as *Columbella rustica* do not develop imposex even under high-TBT exposure conditions although other, closely-related snails from the same genus do (Gibbs et al. 1997).

In summary, the MoA for induction of imposex by TBT has not yet been definitively settled, and it may be that TBT in fact has several or distinct MoAs in different species. This assumption is supported by recent findings of differences in steroid metabolism even between closely related species of snails (Lyssimachou et al, 2009): The androgen precursor androstenedione was mainly converted to 5α-dihydrotestosterone by microsomal fractions isolated from *Bolinus brandaris*, whereas it was primarily metabolized to testosterone by *Hexaplex trunculus*. *B. brandaris* is more susceptible to imposex development than *H. trunculus* and this is related by the authors to the the different androstenedione conversion pathways among the two species. Aromatase inhibition is one hypothesis which remains to be further investigated.

The vertebrate-type steroid and neuropeptide hypotheses are not necessarily mutually contradictory but may simply address different aspects of the prosobranch endocrine system, which may exhibit an even more pronounced similarity to the vertebrate hormonal system. Neuropeptides may act as releasing factors, mediating steroid production and/or metabolism, as proposed by Oberdörster and McClellan-Green (2002) and Oehlmann and Schulte-Oehlmann (2003b), similar to the feedback control of the hypothalamic-pituitary axis in vertebrates. It is also possible that TBT could bind to receptors in the steroid-hormone superfamily (such as RXR), and thereby cause gene transcription that could signal development of the sex organs. These gene products could include neuromodulators such as APGWamide, which again could modulate T and/or E2 production. It appears that no experimental attempts have yet been made to seek a linkage between the three main hypotheses. It may well be that the ongoing genome projects for economically important molluscs (e.g. oysters) or species which are relevant as vectors for human pathogens (e.g. *Biomphalaria glabrata*) will help to understand the MoA of EDCs and other reproductive toxicants in molluscs in due course.

**Endocrine disruption in wild molluscs**

Given that modes of action of EDCs in molluscs are uncertain at present, it could be argued that it is not possible unequivocally to identify cases of endocrine disruption in the field. However, when considered together with the experimental data presented in section 0, there are two examples of substances (organotins and estrogens) which are likely to have caused effects on wild molluscs by means of endocrine disruption, and these are described below. As a further note of caution, it should also be borne in mind that common trematode parasites are able to mimic the effects of some EDCs in molluscs (Morley, 2006, 2008), in particular causing endocrine disruption-like changes in reproduction and immune response. In addition, stress on the immune system (eg due to poor nutrition or immunotoxins) can potentially lead to increased body burdens of parasites and subsequent reproductive impairment. Field data, and laboratory data from field-derived molluscs, should therefore always be interpreted with care.

### 1.7 Effects of tributyltin

The best examples of probable endocrine disrupters where the weight of evidence strongly suggests they have caused serious damage at the invertebrate population and community levels are the triorganotins, of which the most widely distributed and studied is tributyltin (TBT) which was used *inter alia* in many anti-fouling paints. The primary and most potent action of the triorganotins has been against molluscs (both bivalves and gastropods), but there have also been both primary and secondary effects on a range of other invertebrates. A short description of TBT's effects on molluscs is given below, but much more comprehensive information can be found in several reviews, including Fent (1996), Matthiessen and Gibbs (1998) and Matthiessen *et al*. (1999). A discussion of TBT's several putative MOAs in molluscs is presented in section 0.

Tributyltin (TBT)-based antifouling paints for application to the hulls of both large and small vessels were on the market since the early 1960s, but their use only became widespread in the early 1970s when self-polishing copolymer paints were developed (Anderson and Dalley, 1986, cited in Waite *et al*., 1991; Stebbing, 1985). At its height in the 1980s, the market for TBT-based antifouling paints was 2,000-3,000 tonnes p.a. worldwide (DOE, 1986). It gradually became apparent that TBT is one of the most toxic substances to many aquatic species, especially in molluscs where it almost certainly acts as an endocrine disrupter possibly through the induction of high testosterone titres in females (Matthiessen and Gibbs, 1998), although this is still subject to confirmation (see section 3).

Almost all large marine vessels (e.g. 80% of US ships exceeding 4000 tonnes – de Mora, 1996), plus pleasure-craft such as yachts and other marine structures such as salmon cages, were treated with TBT-based paints during the period up to the mid-1980s when many governments brought in bans on their use on pleasure-craft. It was originally thought that the leaching of TBT from large vessels was not so environmentally damaging as from pleasure craft due to the generally greater dilution available in deep water, but this eventually proved not to be the case. However, new uses on large vessels (>25 m) were only stopped in 2003 by the International Maritime Organisation (IMO, 2001), and TBT-containing paint films applied before the 2003 ban did not have to be removed until 17 September 2008.

The field evidence concerns two mollusc groups, the prosobranchs and the bivalves.

### 1.7.1 Prosobranch molluscs

It was noticed in 1970 that female dogwhelk *Nucella lapillus* in the vicinity of Plymouth harbour, UK, possessed a penis (Blaber, 1970). This was subsequently termed 'imposex' by Smith (1971), and associated with exposure to TBT (Bryan *et al*., 1986; Gibbs and Bryan, 1986; Gibbs and Bryan, 1996). TBT causes masculinisation of the female reproductive tract which in the worst cases can lead to sterilization and ultimately to blockage of the oviduct and death due to build-up of unshed eggs. Imposex in *N. lapillus* was and is detectable around many coastlines, but especially near marinas and harbours. It was initially noticed that many populations in southern Britain were sterilised, and subsequently became extinct (Gibbs and Bryan, 1996). We now know that similar effects have been seen in marine prosobranch gastropods around the world, and Matthiessen *et al*. (1999) reported that about 150 species are known to have been affected in this way. TBT's impacts on molluscs and biodiversity in general are thus truly global.

Imposex in dogwhelks and other prosobranchs led to population declines. For example, Gibbs and Bryan (1996) surveyed 79 coastal sites in the UK, between 1986 and 1989. Dogwhelks had become extinct due to TBT at 18% of sites, and more than 50% of females were sterile at another 34% of sites. They live for about 10 years, so populations were slow to decline, and the first extinctions probably did not occur until the late 1980s. Data from Hawkins *et al*. (2002) show that populations with >50% sterility had declined in abundance by approximately 50%, whereas there had been little apparent decline in populations where sterility was <50%. Overall, dogwhelk populations in southern Britain probably declined by at least 50% at 52% of sites. In comparison, Harding *et al*. (1999) surveyed dogwhelks (either wild, or transplanted in locations where the population was already extinct) along the entire UK and continental coastline of the North Sea and English Channel and showed that those populations classed as Category C or D (reduced or terminated egg production) were present at about 65% of sites. It is reasonable to assume that these populations were in decline. In other words, at least half of the dogwhelk populations in northwest Europe had either disappeared or had seriously declined due to TBT. Similar effects were seen in other regions – for example, ivory shell *Babylonia japonica* populations in Japan declined significantly approximately 2 years after TBT-related imposex was first observed (Horiguchi *et al*., 2006). Although Franc (1940; 1952) formerly had no problems in capturing hundreds of egg capsules and also large numbers of adult *Ocinebrina aciculata* on the north coast of Brittany (France), Oehlmann *et al*. (1996) reported a serious population decline for this species between the Bay of Arcachon and Luc-sur-Mer (north of Bayeux) over a period of several years.

Imposex and related effects of TBT (including 'intersex' involving masculinised testicular tissue but no penis growth) have been recorded in many other prosobranchs including American mud snails *Ilyanassa obsoleta* (Bryan *et al*., 1989), European edible whelks *Buccinum undatum* (e.g. Ten Hallers-Tjabbes *et al*., 1994), periwinkles *Littorina littorea* (e.g. Matthiessen *et al*., 1995), spireshells *Hydrobia ulvae* (Schulte-Oehlmann *et al*., 1997), Japanese rockshells *Thais clavigera* (e.g. Horiguchi *et al*., 1994) and Japanese giant abalone (Horiguchi *et al*., 2000). Prosobranch populations which were formerly found in many marine locations around the world, were locally extinguished or depressed by TBT. After the partial TBT bans in the mid-1980s, partial recovery of those species such as *N. lapillus* which lack a planktonic dispersal stage has been relatively slow, and many populations near large harbours are still returning to normal (e.g. Jorundsdottir *et al*., 2005); in some cases the impacts of TBT have remained persistent for over 20 years (Gibbs 2009). This 20-35 year impact of TBT on important rocky shore species almost certainly had secondary consequences for their prey or predators, but such impacts have only been recorded rarely (Spence, 1989; Spence *et al*., 1990).

### 1.7.2 Bivalves

The endocrine disrupting effects of TBT on bivalve molluscs have not been researched as much as in prosobranchs. However, it seems likely that the collapse of native oyster *Ostrea edulis* populations in the UK and elsewhere was partly due to TBT, although other factors such as weather and parasitism also

contributed. One of the largest *O. edulis* populations was in the Crouch estuary in the UK. The average population density in 1986 was only about 0.02-0.03 per $m^2$ (Thain and Waldock, 1986) but this had been 100 times higher in 1957 (2-3 per $m^2$ =18.5 million individuals). The huge decrease in numbers between 1957 and 1986 was principally attributed to the harsh winter of 1962/63, but it is likely that the poor recovery by 1986/87 was at least partly caused by TBT – a few old adults were present in 1987 but were not breeding, and it is known that TBT interferes with breeding in this species. Experiments by Thain and Waldock (1986) clearly showed that TBT at concentrations that were once found in many estuaries (240 ng/l) halted differentiation into females and prevented release of larvae. This was supported by field observations. *O. edulis* only occurred 'sporadically' in trawl surveys of the Crouch in 1987 (Rees *et al*., 2001), and at the most contaminated Crouch station was still only found at 13 per 500 $m^2$ trawl in 1992 (= 0.03 per $m^2$), although many of these individuals were young, indicating that breeding had resumed. However, oyster density had reached 110 per trawl at that same station by 1997 (= 0.22 per $m^2$), a seven-fold increase. In other words, 10 years after most new inputs of TBT had ceased, partial recovery of the native oyster population had occurred.

A more well-known effect of TBT on bivalves was the widespread appearance of shell-thickening in cultured Pacific oysters *Crassostrea gigas*. The best data are for the *C. gigas* fishery in Bassin d'Arcachon near Bordeaux on France's Atlantic coast (Alzieu, 1991; 1998; 2001). The enclosed bay produced 10,000-15,000 tonnes of *C. gigas* annually in the 1970s and from the mid-1980s onwards, but the production grounds were surrounded by 19 marinas and other yacht mooring facilities where TBT began to be used in the early 1970s. From 1975 to 1982, the phenomenon of shell thickening and reduced deposition of larvae (spat) seriously damaged production which dropped to only 3000 t in 1981, and French scientists were the first in the world to link these phenomena with TBT. It is uncertain whether shell-thickening is a form of endocrine disruption, but the reduced larval production was probably caused by similar effects to those observed by Thain and Waldock (1986) in *O. edulis*.

There are no field data which prove unequivocally that endocrine disruption by TBT has affected other bivalve populations. However, there is circumstantial evidence that populations of peppery furrow shell *Scrobicularia plana* in some estuaries have been damaged by TBT (Langston and Burt, 1991; Ruiz *et al.,* 1995b). Furthermore, Minchin *et al*. (1987) showed that settlement of king scallop *Pecten maximus* and several other bivalves failed completely in the North Water of Mulroy Bay on the north coast of Ireland about 2 years after TBT was introduced as a treatment for salmon nets. *P. maximus* settlement recovered in 1986, the year after the use of TBT on salmon farm nets was stopped in Ireland. Estimated population size of *P. maximus* in North Water declined from 554,000 in 1980 to 171,000 in 1985, with the first three year classes being almost absent in 1985, so it appears that reduced settlement fed through to an effect on the population. This was associated with a TBT concentration of 0.75 µg/g wet wt. (as tributyltin oxide) in *P. maximus* tissue from North Water in 1986.

Additional circumstantial evidence for TBT's effects in bivalves comes from studies of clams *Mya arenaria* in a TBT-contaminated part of the St. Lawrence estuary (Gagné *et al*., 2003; Siah *et al*., 2003). These showed that the sex ratio was significantly skewed towards males by comparison with two reference sites, gonado-somatic index and progesterone titres were reduced, and sexual maturation was delayed. Furthermore, the presence of vitellin-like proteins in female gonads, and the capacity of females to produce E2, were both reduced in the TBT-contaminated area, indicating the existence of a masculinising influence. Somewhat similar effects have been observed in a mussel *Mytilus edulis* population near a naval dockyard (Hellou *et al*., 2003) which would have been a source of TBT, but it is uncertain whether organotins were the causative factor.

Finally, observations of the benthos in the Crouch estuary, U.K., made after 1987 when TBT was banned from use on small boats, showed a strong association between the decline of TBT contamination and an increase in abundance and diversity of the bivalve (and prosobranch) community (Rees *et al*. 1999, 2001; Waldock *et al*., 1999). There were also TBT-related changes in certain non-mollusc species (e.g. sea squirts), some of which might have been a secondary consequence of the impacts on molluscs.

In summary, organotins have done great damage to both prosobranch and bivalve populations around the world, and there seems little doubt that endocrine disrupting mechanisms are at least partly responsible.

### 1.8 Effects of estrogens and their mimics

Following the discovery of widespread feminisation of male fish by natural and synthetic estrogens (see Matthiessen 2006 for a review of the situation in the UK), there has been a search for analogous endocrine disruption in, *inter alia*, mollusc populations. The weight of evidence for population effects of estrogens in molluscs is, however, much weaker than for the effects of TBT. Binelli *et al*. (2001; 2004) reported possible endocrine disruption in zebra mussels *Dreissena polymorpha* exposed in Pallanza Bay on Lake Maggiore to *pp'*DDT and its metabolites originating from a chemical factory on the River Marmazza. *D. polymorpha* from Pallanza Bay released their oocytes earlier than those from a nearby reference site, and 40% contained degenerating oocytes, compared with only 10% at the reference. Furthermore, the date of first sperm release from males was 2 months later than oocyte release in females, although mature sperm were present in the testes. These observations were associated with body burdens in the mussels of 300-900 ng pp'DDE/g lipid, and 100-400 ng op'DDT/g lipid, both of which DDT homologues are known to have estrogenic effects in fish. However, it is not certain that these effects occurred by an endocrine disrupting mechanism, and it should be noted that no abnormal ovotestis (i.e. oocytes in the testis of this normally gonochoristic [separate sexes] species) was observed.

More recently, Chesman and Langston (2006) have reported the presence of male ovotestis in 17 out of 23 populations of the normally gonochoristic bivalve *Scrobicularia plana* in estuaries in southwest England. This mainly appears to occur in the summer months when gametogenesis takes place, and is accompanied by a sex ratio skewed towards females. Langston *et al.* (2007) confirmed these findings, and also showed that oocyte size increased in both females and ovotestis-containing males in areas where ovotestis prevalence was high (up to 60% of males showed ovotestis). There appeared to be an association between the occurrence of male ovotestis and the degree of human influence in a given estuary, but no analyses were conducted for EDCs, and some ovotestis was present even in estuaries considered to be relatively free of sewage and industrial discharges. However, experimental exposure of undifferentiated *S. plana* to estrogen-contaminated sediment for 1 month (followed by 4 months recovery in a relatively uncontaminated estuary) produced 44% of males with ovotestis when they matured, compared to 0-8% in controls. Oocyte diameters were also increased in the exposed animals. The estrogens used were a mixture of E2 and ethinylestradiol (EE2) (both at 100 µg/kg wet wt.) and nonyl- and octylphenol (NP and OP) (both at 1000 µg/kg wet wt.). These concentrations are rather high compared with those in most estuarine environments, implying the need for further experimental work with *S. plana*, but there seems little doubt that the effects described by Langston *et al*. (2007) are indeed a form of endocrine disruption.

Blaise *et al*. (1999) have identified that the clam *Mya arenaria* contains a vitellin-like protein that can be indirectly determined by measuring alkali-labile phosphate (ALP). The ALP is induced by injections of E2, NP or pentachlorophenol (PCP), and a survey of *M. arenaria* in the Saguenay Fjord, Quebec, showed significant variations in induction of ALP along the fjord. This was accompanied by delayed gametogenesis in the upper part of the fjord (Gauthier-Clerc *et al*., 2002), and it was hypothesised that there was a persistent dysfunction of vitellogenesis which may have been caused by anti-estrogens. Similar induction of vitellogenin-like protein, again in terms of ALP, has been observed more recently in clams (*Tapes philippinarum* and *Cerastoderma glaucum*) sampled from estrogen-contaminated areas near a sewage discharge in the Venice Lagoon (Matozzo and Marin, 2007). It was noted that the ALP response was greater in June (the pre-spawning period) than in January (the stage of early gametogenesis).

Finally, Gagné and Blaise (2003) have shown that freshwater mussels *Elliptio complanata* held downstream of a municipal effluent discharge for 90 days experienced reduced serotonin and increased monoamine oxidase activities, both of which are involved in sexual differentiation. Similar effects could be induced by the injection of E2 or NP, suggesting that the estrogens and their mimics present in sewage effluent may be affecting reproduction in freshwater bivalves.

There have been no other reports from the field of possible estrogenic endocrine disruption in molluscs. However, when the limited field data are compared with the experimental data described in section 0, it seems possible that estrogens are affecting wild molluscs in some places, and that these effects are a form of interference with the hormone system which remains to be fully explained.

### 1.9 Summary of EDC effects in wild molluscs

It is well-established that the organotins have caused widespread indirect masculinising effects in molluscs which have damaged populations of both prosobranchs and bivalves, and which are a form of endocrine disruption, although the precise mode(s) of action has not been completely clarified. These effects were not predicted by environmental risk assessment procedures, but revealed by field observations, and have been confirmed by laboratory-based experimentation (section 0).

There is much less evidence for estrogenic effects in wild molluscs, although experimental studies suggest that such effects are likely. Unfortunately, modes of action of estrogens in molluscs are only speculative at present. However, the induction of male ovotestis in wild populations of the gonochoristic bivalve *S. plana* (Langston *et al*., 2007), and of ALP in wild clams living near estrogenic sewage discharges (Matozzo and Marin, 2007) seem to be a clear effects of this type. It appears likely that such effects may be associated with long-term interference with reproductive success if sufficiently severe, but data on this point are lacking. A more thorough understanding of general life-history and husbandry characteristics in molluscs must be obtained before an effect of a chemical in the field can be clearly ascribed.

**Experimental induction of endocrine disruption in molluscs**

Despite the evidence for endocrine disruption in wild molluscs caused by organotins and estrogens, experimental data are essential for its interpretation. This section therefore describes what is known about endocrine disruption in molluscs exposed to these EDCs under controlled conditions, and also includes an assessment of the more limited experimental data on other types of EDC.

## 1.10 Organotins

Organotins were discovered in the late 1950s to be powerful biocides. In the 1960s, substances like triphenyltin (TPT) became widely used as agricultural fungicides, and tributyltin (TBT) began to be used on boat hulls as an antifouling compound, and on timber as a preservative. They were found to be particularly acutely toxic to molluscs (Floch *et al.,* 1964; Frick and Dejimenez, 1964; Ritchie *et al.,* 1964, 1974; Deschiens *et al*., 1965, 1966; Hopf *et al*., 1967; Deschiens and Floch, 1968; Da Souza and Paulini, 1969; Chu, 1976; Smith *et al*., 1979), with short-term LC50 values in the range 10-300 µg/l, and longer-term lethality in the range 0.001-7.0 µg/l (Cardarelli, 1973; Ritchie *et al*., 1974). Organotins such as bis (tri-n-butyltin) oxide consequently also entered use in the 1960s as molluscicides for the control of the aquatic snails which transmit bilharzia (schistosomiasis).

The endocrine-disrupting properties of organotins in molluscs were only gradually discovered in the 1980s, and fully recognised in the 1990s (ACP, 1994; Fent, 1996; Matthiessen and Gibbs, 1998; Maguire, 2000). In particular, Bryan *et al*. (1987, 1988) showed that concentrations of TBT as low as 2.5 ng/l caused imposex in female dogwhelks *Nucella lapillus*, characterised by the growth of a penis and (in severe cases) blockage of the oviduct and death. Since then, similar effects (including the closely related phenomenon of intersex in littorinids – Bauer *et al*., 1995; Matthiessen *et al*., 1995) have been induced by TBT-exposure in many other species of prosobranch mollusc (reviewed by Oehlmann *et al*., 2007). Masculinization of female abalone has also been caused by TBT and/or TPT (eg., Horiguchi *et al*., 2000, 2002, 2005). Furthermore, it appears that freshwater gastropods (and freshwater species in general) may be less sensitive than their marine relatives (Leung *et al*., 2004; 2007), although there are insufficient freshwater data to be sure if there is a real difference in sensitivity. The test species proposed in subsequent chapters (*Lymnaea stagnalis* and *Potamopyrgus antipodarum*) are both freshwater organisms, but are nevertheless considered appropriate for further investigation since they tolerate salinities up to 7‰ and 15‰, respectively (Glöer *et al*., 1992; Jacobsen and Forbes, 1997). Although less research has been conducted on TPT, this also causes imposex in gastropods at similarly low concentrations to TBT (e.g. Horiguchi *et al*., 1997; Schulte-Oehlmann *et al*., 2000).

Whether or not imposex or intersex occur as a result of organotin exposure in particular prosobranch species, the net result is generally manifested as reduced reproductive success, and it seems likely that these reproductive effects, which generally occur in the ng/l range, are all the result of endocrine disruption. TBT's precise mode of endocrine disrupting action remains the subject of debate, and a discussion of this topic is given in section 0, but the fact that organotins interfere with prosobranch endocrine systems in one or more ways is well-established.

There is much less experimental evidence for organotin-induced endocrine disruption in bivalves, and little for such effects in other mollusc groups such as the cephalopods. Although Ortiz and Ré (2006) recently reported the first case of imposex in the Patagonian red octopus *Enteroctopus megalocyathus*, the authors decline to link the phenomenon to TBT-mediated endocrine disruption. Noting organotin sediment

contamination of an industrial harbour in the Nuevo Gulf (Argentina) in the range of 4 µg TBT/kg and imposex incidences in gastropods of about 100 % in the cephalopod fishing area, one might conclude that TBT had also affected octopus in that area. However, the malformation was only seen in one out of 185 females.

It should, however, be noted that absence of evidence is not necessarily evidence of absence, given that prosobranchs have been studied far more intensely than other groups. There is no doubt that TBT at ng/l concentrations can damage reproduction in bivalves. The work of Thain and Waldock (1986) with *Ostrea edulis*, already cited in section 0, showed that TBT exposure at 240 ng/l for 74 days prevented larval release and eliminated animals in the female phase. A higher concentration (2620 ng/l) almost prevented sexual differentiation and shell growth. Similarly, Ruiz *et al*. (1995 a & b) showed that TBT at 250 ng Sn/l produced a significant reduction in the production of embryos by adult *Scrobicularia plana*, and 50-125 ng Sn/l prevented and/or damaged shell growth of the larvae. Work in Japan has also shown that exposure of pearl oyster *Pinctada fucata* and Manila clam *Ruditapes philippinarum* adults to ng/l concentrations of TBT for 1-3 weeks causes abnormal larval development (Inoue *et al*., 2004, 2006).

It is possible that these effects of TBT on the reproduction of bivalves are not related to endocrine disruption. However, several studies have shown that exposure of bivalves to TBT appears to cause steroid hormone imbalances which might be implicated in damaged reproduction. For example, 7 day exposures of clams *Ruditapes decussata* to TBT at concentrations between 100 and 2270 ng Sn/l produced a significant increase in testosterone titres (Morcillo *et al*., 1998). This was associated with a reduced ability of the cytochrome P450 enzyme system in digestive gland microsomes to metabolise testosterone to estrone and estradiol. In related work, Morcillo and Porte (2000) transplanted *R. decussata* to a TBT-contaminated location for 5 weeks and observed a 33% increase in testosterone titres and a 5-fold decrease in estradiol titres. Similar changes in sex hormones have been observed in another species of TBT-exposed clam *Meretrix meretrix* (Wang *et al*., 2005). It should however be noted that the mechanistic relationship of TBT exposure to possible steroid hormone imbalances remains uncertain.

In summary, laboratory exposures of various molluscs to TBT have been able to mimic effects seen in the field in areas of TBT contamination, ranging from induction of biomarkers (e.g. imposex) to impacts on reproduction. Although TBT's mode of action on molluscs is still unclear, it appears well-established that these effects are the result of a form of endocrine disruption.

### 1.11 Estrogens and their mimics

A number of laboratory studies have shown that estrogens and their mimics are capable of interfering with mollusc reproduction. Many of these have been reviewed by Rotchell and Ostrander (2003), Oetken *et al*. (2004), Porte *et al.* (2006), Croll and Wang (2007), Lagadic *et al*. (2007), Weltje and Schulte-Oehlmann (2007), and Oehlmann *et al*. (2007), so only a summary of this information is given below.

One of the earliest studies was that of Mori *et al*. (1969) who showed that if oysters *Crassostrea gigas* are injected with E2 at early stages of maturation, they can be induced to change sex from male to female. Furthermore, Varaksina and Varaksin (1991), Varaksina *et al*. (1992) and Wang and Croll (2004) have shown that injecting scallops with E2 (and other steroids) at later developmental stages can stimulate both oogenesis and spermatogenesis, and can lead to increased gonad weight and larger oocytes.

In the present context, the most interesting work has involved reproduction experiments with various molluscs exposed via the ambient water to estrogens, androgens and anti-androgens. One of the first of these was conducted by Oehlmann *et al*. (2000) who treated the freshwater prosobranch *Marisa cornuarietis* and the marine prosobranch *Nucella lapillus* with the weak estrogen mimics bisphenol A (BPA) and octylphenol (OP) at concentrations between 1 and 100 µg/l for up to 12 months in life-cycle tests (*M. cornuarietis*) and 3 months in tests with adults (*N. lapillus*). In both species, so-called 'super-females' were induced, characterised by enlarged sex organs and stimulation of oocyte production. Effects were reported to be statistically significant at the lowest concentration tested (1 µg/l). Further work with *M.*

*cornuarietis* (Oehlmann *et al*., 2006) derived a no-observed-effect-concentration (NOEC) for super-female induction of 7.9 ng BPA/l, clearly a high level of potency for a so-called weak estrogen. The effect was temperature-dependent (greater at lower temperatures), and could be completely eliminated by co-exposure to estrogen-antagonists, indicating that BPA was probably acting as an estrogen receptor agonist. These results were subsequently disputed following repeated experiments (Forbes *et al*., 2007a & b). Forbes *et al*. (2008a) also tested the effect of temperature on reproductive output of *M. cornuarietis* and, whereas egg production of control snails was reduced at 22$^{o}$C compared to 25$^{o}$C, there was no evidence for a greater stimulation of reproduction by BPA at the lower temperature. However, the repeat studies reported by Forbes and her co-workers did not precisely replicate the test conditions used by Oehlmann and his team, and there is continuing disagreement about interpretation of these results (Dietrich *et al*., 2006; Oehlmann *et al*., 2006b; Oehlmann *et al*., 2008; Forbes *et al*. 2008b). The degree of estrogenicity shown by BPA in prosobranchs is therefore still open to question, although the super-female phenomenon itself seems to be genuine, as similar effects have also been seen by other research groups in other species (see below). Perhaps the most important lesson to be learned from these conflicting results is the profound influence of test conditions (particularly temperature, life history and general husbandry characteristics) in experiments of this type, an issue which must be taken seriously if OECD is to develop long-term test guidelines with molluscs.

The stimulation of reproductive output by estrogen exposure reported by Oehlmann *et al*. (2000; 2006) for *M. cornuarietis* also occurs in another prosobranch, the parthenogenetic and ovoviviparous freshwater species *Potamopyrgus antipodarum* (Duft *et al*., 2003b). In these experiments, the test organisms were exposed in spiked sediments to BPA and OP, and in both cases, the lowest-observed-effect-concentration (LOEC) for an increase in the number of embryos after 8 weeks exposure was 1 µg/kg dry wt., the lowest concentration tested. NP was slightly less potent, with an 8 week LOEC of 10 µg/kg dry wt. EE2 was used at 30 µg/kg dry wt. as a positive estrogenic control, and also induced the production of additional embryos. Experiments with *P. antipodarum* have also been conducted where dosing was via the ambient water (Jobling *et al*., 2004). These revealed increased embryo production after 2-3 week exposures to EE2 (LOEC 1 ng/l), BPA (LOEC 1 µg/l), and OP (LOEC 5 µg/l). Similar effects were seen when *P. antipodarum* was exposed to dilutions of treated but estrogenic sewage effluent. Unlike *M. cornuarietis*, *P. antipodarum* tolerates a wide range of temperatures (0-34$^{o}$C; Benson and Kipp, 2009), and it would be worthwhile investigating whether temperature affects sensitivity to estrogens within this range.

Yet other experiments with EE2 have been conducted with the freshwater pulmonate gastropod *Lymnaea stagnalis*, and in this species also, increased egg laying was observed at the (very high) concentration of 500 ng/l, together with reduced growth, delayed hatching and induction of a vitellin-like protein at concentrations down to 50 ng/l (Segner *et al*., 2003).

Recently, as-yet-unpublished experiments have been conducted in which populations of the hermaphroditic pulmonate *Planorbarius corneus* were exposed for 14 weeks to dilutions of an estrogenic sewage effluent in mesocosm enclosures and allowed to reproduce (Clarke *et al*., 2009). Numbers of egg masses and egg weights were recorded every 2 weeks. There was a concentration-related and statistically significant increase in the cumulative numbers of egg masses produced per snail in undiluted and 50% effluent, an observation which is consistent with the effects produced by estrogens in *M. cornuarietis*, *P. antipodarum*, and *L. stagnalis* (see above). In a laboratory experiment, developmental mortality was seen in the F1 offspring of *P. corneus* exposed for a year to a mixture of estrogenic hormones (EE2, E2 and E1) and estrogen hormone mimics (NP, OP and BPA) similar to that found in sewage effluent, and gametogenesis and successful reproduction was also disrupted in the survivors. Thus it seems that so-called super-feminisation should not be construed as a beneficial effect.

Further evidence of the importance of test conditions is provided by recent unpublished experiments (Dr. Ed Routledge pers. comm., 2008) in which *P. corneus* adults were exposed to E2 at 100 ng/l for 26 days in either cool or warm conditions. E2 had no effect on egg production in the warm tanks, but in the cool tanks, egg production was slowed in the controls and restored to normal in the exposed snails. In other

words, seasonal changes in temperature, or simply poor control of test conditions, can have a major influence on the responses of gastropods to an estrogen.

It is noteworthy that in several of the experiments described above, the dose-response curves were in the shape of an inverted 'U', with no effects at low concentrations, excess embryos produced at intermediate concentrations, and embryo production being inhibited at higher concentrations. This type of non-linear response will also have to be accounted for when designing test guidelines.

Few full life-cycle tests with gastropod molluscs exposed to estrogens have yet been conducted. However, Czech *et al*. (2001) exposed *L. stagnalis* for 7-12 weeks to NP, and although fecundity was decreased at 100 µg/l (NOEC 10 µg/l), no effects were seen in the F1 offspring. Czech *et al*. (2001) did not believe that the effect on fecundity was caused by endocrine disruption because there was severe histopathological damage to several tissues, indicating a deleterious effect on general health.

Finally, few estrogen-exposure experiments have been conducted with bivalves in addition to those associated with field studies and described in section 0. Lavado *et al*. (2006) studied the effects of 3 week exposures of a mixture of crude oil and alkylated phenols (unspecified) on adult *Mytilis edulis*. This had clear effects on steroid metabolism, producing increases of esterified E2 and T in the gonads, and an increase in estradiol sulphotransferase activity. Nice *et al*. (2000) exposed oyster *Crassostrea gigas* embryos to NP for up to 72 hours, and showed that development towards the normal D-larval stage was delayed by all test concentrations down to 0.1 µg/l (unbounded LOEC), although the effect only persisted to 72 h at higher concentrations. 100 µg/l caused deformities of the D-larvae, but it is not known whether these effects were caused by endocrine disruption. Of more interest is the subsequent study (Nice *et al*., 2003), in which *C. gigas* D-larvae were exposed to NP for just 2 days, between days 7 and 8 post-fertilisation, and then allowed to mature under controlled, NP-free conditions for 10 months. 17% of the adults at the lowest test concentration (1 µg/l) were found to be fully functional hermaphrodites (i.e. self-fertile), a highly unusual condition in this species, and the adult sex ratio was skewed towards females. Furthermore, the survival of the F1 offspring of the adults was very poor. It seems highly likely that the interference with sexual development, combined with the trans-generational effects, was caused by endocrine disruption.

The data described in this section show that the reproduction of some molluscs is sensitive to estrogens and their mimics, in some cases at environmentally-relevant concentrations. This supports the limited data suggesting that estrogens may be affecting some mollusc species in the field.

### 1.12 Androgens, their mimics and antagonists

Relatively little information is available about molluscan responses to EDCs which interact with the androgen receptor in vertebrates. As with the estrogen receptor, the presence and role of an androgen receptor in molluscs is still the subject of research. However, Tillmann *et al*. (2001) reported experiments with the prosobranchs *M. cornuarietis*, *N. lapillus* and *Nassarius reticulatus* exposed to the anti-androgens cyproterone acetate (1.25 mg/l) and vinclozolin (0.03-1.0 µg/l). In 5-12 month experiments, it was shown that the anti-androgens significantly reduced penis and accessory male sex organ sizes, and were able to antagonise androgen-mediated responses such as imposex development. The direct effects of the anti-androgens were considered mild in comparison with the effects of the estrogen EE2, and the androgens TBT and methyltestosterone (MT). In the case of MT, 0.5 µg/l caused an enhancement of imposex intensity (as measured by the Vas Deferens Sequence Index, or VDSI) after 9 months exposure. On the other hand, life-cycle experiments with *L. stagnalis* exposed to the androgens β-sitosterol (1-100 ng/l) and t-methyltestosterone (1-100 ng/l) revealed atrophy of the albumen gland, but there was no effect on fecundity, hatching rate, or fertility, and no effect on the F1 generation (Czech *et al*., 2001).

### 1.13 Others EDCs

Almost nothing is known about the possible role in molluscs of EDCs other than organotins, and sex steroid-like xeno-estrogens, xeno-androgens and androgen antagonists. However, several endocrinologically-active tissues are present (e.g. nervous system, kidney and midgut gland – see section 0), and these may well be responsive to other modes of disruption. One of the few studies to investigate this issue (Frouin *et al*., 2007) studied the effects of dietary and sediment-associated polycyclic aromatic hydrocarbons (PAH) on clams *Mya arenaria*. These 30 day exposures produced a variety of effects including immune suppression (reduced phagocytosis) and oxidative stress (lipid peroxidation), and resulted in delayed gametogenesis in both sexes. It was suspected that this effect resulted at least partially from the oxidative stress which may have damaged steroid (corticoid hormone?)-synthesising cells, although no direct evidence for this was presented. It is also worth noting that certain types of hormonally-active pharmaceuticals (eg fluoxetine and other types of serotonin re-uptake inhibitors) appear to be able to affect bivalve mollusc reproduction, although modes of action are unclear (Fong, 1998; Fong *et al*., 1998).

### 1.14 Summary

There is good evidence that both organotins and estrogens at ng/l concentrations are able to interfere with normal reproduction in prosobranchs and bivalves, and these effects have been associated with imbalances in steroid hormones, although the precise mode(s) of action are not fully understood (see section 0). There is more limited evidence for the impact of androgen-receptor agonists and antagonists on sexual development and reproduction in some molluscs. Finally, there is weak evidence that some polycyclic aromatic hydrocarbons (PAH) may be able to cause delays in gametogenesis through damaged steroid metabolism.

When these experimental data are considered together with field observations in both prosobranchs and bivalves (section 0), it is apparent that they help to explain many of the presumed impacts of organotins and estrogens on mollusc populations that have been reported. Overall, there is virtually no doubt that endocrine disruption occurs in some molluscs, and that it is able to damage reproduction and even cause population declines.

It will be apparent that there is considerable experience in conducting partial life cycle reproduction experiments with prosobranchs and bivalves exposed to EDCs, and more limited experience with full life cycle experiments. In some cases, the test organisms are capable of being cultured in the laboratory, while other test species at present have to be collected from the field. There is thus a good database of information and pool of expertise from which standardised test protocols can be drawn. This issue will be considered in detail in sections 0 and 0.

**Experimental design considerations**

### 1.15 Exposure duration

A general assumption in ecotoxicology is that the intensity of biological effects caused by a chemical is a function of the exposure level (i.e. concentration of the chemical) and the exposure time (i.e. duration of the test). Consequently, an increased exposure time should result in appearance of effects at lower concentrations (OECD, 2008). Therefore, full life cycle (FLC) testing of a chemical will potentially produce effects at lower concentrations compared to a partial life cycle (PLC) test with the same species. However, this supposed advantage is counterbalanced by a number of other factors. These are presented in section 1.15.1 before general aspects of PLC and FLC tests with molluscs are discussed in sections 1.15.2 and 1.15.3. Candidate PLC and FLC tests methods with molluscs are outlined in section 0.

#### 1.15.1 Strength and weaknesses of partial versus full life cycle tests

Because endogenous hormones regulate various physiological functions during development, growth and reproduction, EDCs can interfere with processes like sexual differentiation and maturation, reproduction (including gametogenesis and fertilisation), embryonic development, juvenile growth and aging. The chances of identifying the potential impact of a test compound on one of these processes increase with the number of sensitive or critical phases and the number of endpoints being considered in the test design. For fish it has been shown that the toxicity of a significant number of organic chemicals and metals tested in FLC studies could not be predicted from the results of an early life stage exposure (reviewed in OECD, 2008). As there is clearly less experience with FLC tests in molluscs, the relevance of these findings for this invertebrate group cannot be judged reliably but the assumption is convincing from a theoretical point of view. However, the few experimental examples allowing a direct comparison of the sensitivity of molluscs to EDCs in PLC and FLC tests provide conflicting results (cf. section 1.21.2).

The majority of PLC studies with molluscs expose reproducing adults. This part of the life cycle is not only considered to be particularly sensitive to EDCs but any changes in reproductive output may have direct population level consequences. Furthermore, fecundity parameters like the number of produced clutches, eggs or embryos are comparatively easy to measure. The main strength of PLC tests is the reduced time to conduct the test compared with an FLC test with the same species. This also reduces the costs and especially the danger of losing the organisms to disease before the experiment is terminated, of technical failures resulting in a die off of test organisms, and of interrupted or decreasing exposure which can also be caused due to excessive degradation of the test compound by microbial growth. These disadvantages of FLC studies can at least in part be compensated by choosing faster maturing species which reduces study duration. Nevertheless it can be argued that such species might also be less sensitive than molluscs with a longer generation time. Because the exposure duration of a study is a compromise between the time necessary to elicit an effect and the appropriate duration to control costs and reduce the danger of system failures, PLC studies should not be disregarded *per se* as long as they prove to be sensitive enough for the identification and hazard assessment of EDCs.

The obvious weakness of PLC studies is that they do not expose all life stages. Therefore, these tests do not provide data on potential effects if the most sensitive part of the life cycle is excluded or if there is a concern about trans-generational effects which can only be identified in FLC or even multigeneration exposures. In contrast, an FLC design ensures that all life stages are exposed and that concentrations causing effects at the population level can be predicted with higher probability.

As discussed in section 1.1, the development and validation of new toxicity testing guidelines with molluscs focuses on Level 5 of the Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals (Gourmelon and Ahtiainen, 2007; OECD, 2004a). This requires apical *in vivo* tests sensitive to both endocrine and non-endocrine mechanisms to be used in risk assessment. These tests will not necessarily be specific for EDCs or a particular MOA but this does not restrict their suitability for an assessment of the potential ecological risk posed by an analysed chemical. However, as an additional option they have the potential to include histopathological endpoints and biomarkers to examine:

- whether the observed effect of the test compounds is a result of its general toxicity or due to a more specific and potentially endocrine mechanism and
- whether fecundity alterations are associated with histological changes in the gonads.

### 1.15.2 Partial life cycle (PLC) tests

PLC studies can be performed with adult, sexually mature molluscs covering the period of reproduction or are initiated with fertilised eggs, embryos or juveniles covering the period until sexual maturity and measuring various developmental endpoints (cf. section 1.21.1). There are less examples of the latter approach (e.g. Tillmann et al., 2001; Nice et al., 2003) so that the focus here will be on PLC designs with sexually mature molluscs.

The 'reproduction test' as the most commonly used type of PLC study with molluscs assesses the reproductive output or fecundity of exposed adults by counting the number of produced clutches and eggs or embryos (e.g. Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2000; Jumel et al., 2002; Duft et al., 2003a & b, 2007; Jobling et al., 2004; Coutellec et al., 2008; Mazurová et al., 2008; Schmitt et al., 2008). Although for pragmatic reasons there is a certain interest to minimise test duration, the exposure period should be several weeks to ensure that it covers gametogenesis and spawning (in oviparous species: majority of gastropods and bivalves) or embryo development (in ovoviviparous species, e.g. *Potamopyrgus antipodarum* and *Viviparus viviparus*). It has to be considered that female neogastropods, mainly a marine group, but also pulmonates have in particular developed specialised sections in the oviduct during evolution to store and nourish sperm after copulation for a period of weeks to months (Fretter and Graham, 1962). This characteristic limits their use in reproduction tests because potential effects of test chemicals on spermatogenesis cannot be identified except by histopathological testis analyses or if the exposure starts with sub-adults that are still sexually immature.

An ideal test species for a reproduction test should exhibit a steady reproductive output throughout the year. However, most molluscs like other invertebrates and fish from temperate zones and even some (sub-)tropical species are seasonally reproductive. The seasonal pattern is synchronised by external triggers, with day length and temperature (mostly continuous season-related changes in temperate species and fast declines in tropical and subtropical molluscs indicating the beginning of the rainy season) being the most important. Even under controlled conditions in the laboratory without external triggering, molluscs may retain a seasonal pattern in reproduction over long time periods. For obvious reasons, molluscs with one single or several consecutive spawning periods, typically in spring/early summer, followed by complete sexual repose for the rest of the year (e.g. most marine neogastropods and bivalves), are of limited practical relevance for this type of test. Although the candidate species for PLC and FLC tests presented in section 0 do exhibit a seasonal reproductive pattern with a phase of higher output, they never cease to reproduce during the rest of the year and can therefore be used in reproduction tests. Even for *Crassostrea gigas,* spawning throughout the year can be achieved at water temperatures around 19°C (Fabioux et al., 2005). However, seasonally varying fecundity has to be taken into account when interpreting the results of tests with EDCs.

The current candidate protocol for the PLC test with *P. antipodarum* in section 1.21.1 covers a test duration of 8 weeks (56 days) with an assessment of embryo numbers in the brood pouch of 10 snails per replicate after 28 days and of all surviving snails at the end of the experiment (day 56). Additional

parameters such as the counting of released offspring may offer the advantage of a better coverage of the reproductive output over time but the practicability of this approach has still to be investigated. Furthermore, this approach would require longer test durations to ensure that all newborn snails have developed during exposure to the test compound. On the contrary it would be desirable to reduce the test duration from 8 to 4 weeks without substantial loss of test sensitivity (Duft et al., 2007; Gourmelon and Ahtiainen, 2007) but this possibility is also still to be investigated. According to Zinßmeister (2006) and Dorgelo (1991) the average development time of *Potamopyrgus* embryos is 21 days at l6°C and 30 to 35 days at 15°C, respectively. Zinßmeister (2006) observed a shell on developmental day 8. Based on these results a reduced exposure time of 4 weeks seems to be feasible for the PLC test with *P. antipodarum* because it still guarantees that all embryos found in the brood pouch at the end of the test will have developed *ex ovo* under exposure to the test chemical at 16°C.

A further PLC protocol has been proposed by colleagues from INRA (Rennes, France) for *L. stagnalis* which is provided in Appendix 2 of this document and based on a paper that has not yet been peer-reviewed. This test covers an exposure period of 28 days, starting with young adults (snails that reached maturity and began to reproduce during the test), so that effects of the test compound on spermatogenesis can be assessed. Fertility and fecundity are used as test endpoints. Additional parameters such as hatching rate or developmental duration ($HT_{50}$) can also be assessed. This protocol remains to be optimized and validated.

Additionally to fecundity, further parameters can be measured during PLC tests, e.g. gonad histopathology or vitellin induction (cf. section 1.21.3). These are clearly not essential but may help to resolve the potential MoA of the test compound, although it should be emphasised that the MoA of EDCs have not yet been definitively established in any molluscs.

### 1.15.3 Full life cycle (FLC) tests

Ecotoxicological FLC studies with molluscs have been conducted with only few species so far, including the prosobranch snail *Marisa cornuarietis* (Oehlmann et al., 2000; Forbes et al., 2008b), the pulmonate *Lymnaea stagnalis* (Czech et al., 2001; Weltje et al., 2003; Ducrot et al., 2008a, b) and the oyster *Crassostrea gigas* (Nice et al., 2003). Despite the limited experience with these tests, *Lymnaea* has been widely used for short term and chronic ecotoxicological testing and *Crassostrea* is an important aquaculture species so that favourable conditions for growth and reproduction are well known and promising candidate protocols can be proposed for both species (cf. section 1.23). In contrast, *M. cornuarietis* is characterised by a number of disadvantages (Duft et al., 2007) so that this species seems to be less suited for a standardised FLC test.

The proposed protocols for *L. stagnalis* and *C. gigas* cover exposure periods of 31 weeks (217 days) and 40 weeks (280 days), respectively. Such time scales are challenging and logistically complex for many laboratories because long-term maintenance of exposure systems requires extensive experience that is not widely available. Furthermore, test conditions specified in detail in section 0 have been derived by combining information from a number of publications on short-term tests with both species. Therefore, the feasibility of the FLC protocols should be demonstrated before any validation exercises.

A further aspect to be investigated is the potential extension of the existing PLC protocol with *P. antipodarum* to an FLC test design. Given that the time to reach sexual maturity is 3 months in this species, the FLC test would require a duration of 20 weeks if the exposure of adults in the F0 and F1 generation can be limited to 4 weeks as discussed above. Otherwise, the test would require 28 weeks.

## 1.16 Possible periods of exposure during development

Comparably to fish (cf. OECD, 2008), life histories of molluscs can be broadly divided into two ontogenic groupings: those with an 'indirect' development and those with 'direct' development. Although this

distinction is questionable from a scientific point of view (Fioroni, 1992), it is helpful for addressing ecotoxicologically relevant differences. Molluscs with an 'indirect' development are typically oviparous and exhibit five distinct life history periods: embryonic, larval, juvenile, adult, and senescent. The larval period is not necessarily a free-swimming, planktonic phase of the life cycle (like in most bivalves) but can also be passed sheltered as a developmental phase in the developing egg (e.g. in the vast majority of freshwater gastropods and most marine snails in temperate zones) which is often surrounded by a gelatinous mass (egg mass, clutch) or solid egg capsule. The so-called 'direct' development trait in molluscs can be found in the rare cases of ovoviviparous species such as *Viviparus viviparus* and *Potamopyrgus antipodarum*, one of the candidate species for a PLC test. These species are characterised by four life history periods: embryonic, juvenile, adult, and senescent. The larval period is not missing in the strict sense but integrated into the embryonic period. The embryo develops within the egg shell in the maternal organism so that test chemicals might be less bioavailable for the developing embryo in ovoviviparous species compared to the embryonic and larval phases in oviparous molluscs.

Molluscs exhibit multiple reproductive modes, like simultaneous hermaphroditism (both outcrossing and self-fertilization) and consecutive hermaphroditism (mostly protandric with sex change from male to female; more rarely protogynic with sex change from female to male), gonochory and parthenogenesis, each of them combined with semelparity (individuals reproduce only once during their lifetime) or iteroparity (individuals reproduce several times throughout their life). This allows the assessment of contaminant effects on a broad variety of reproductive strategies (Oehlmann and Schulte-Oehlmann, 2003b). Furthermore, molluscs exhibit a large range of life cycle strategies, especially with respect to longevity. While the majority of cephalopods, marine opisthobranch snails and most of freshwater and terrestrial gastropods are short-lived species with a maximum life span of more or less one year, the marine prosobranch snails and many bivalves are long-lived so that they can integrate contamination of their environment over long periods. Approximately 40% of the marine bivalve species and more than 20% of the marine prosobranchs attain maximum ages of more than 14 years according to Heller (1990). For single species even longer life spans have been reported, like for example more than 50 years for the abalone *Haliotis cracherodii* (Powell and Cummins, 1985) and more than 100 years for some marine (220 years for *Arctica islandica* according to Jones, 1983) and freshwater bivalves (116 years for *Margaritifera margaritifera* according to Bauer, 1987).

All test species being considered in this review are comparatively long-lived with a life span of at least two years but represent different reproductive modes. *P. antipodarum*, one candidate species for the PLC test is ovoviviparous (i.e. 'direct' developing) and parthenogenetic. Males have only been observed very rarely in Europe in the field (Wallace, 1979; Ponder, 1988; Gérard et al., 2003) and laboratory populations used for ecotoxicity testing consist exclusively of females (Duft et al., 2007). The two candidate species for FLC tests, *L. stagnalis* and *C. gigas* are both oviparous (i.e. 'indirect' developing) hermaphrodites. *Lymnaea* is a simultaneous hermaphrodite although with a tendency to protandry (in the ovotestis, sperm are produced earlier than oocytes) and an *in ovo* larval development while *Crassostrea* is a consecutive protandric hermaphrodite with a free-swimming larval phase (for details cf. section 1.23).

Although it can be argued from a theoretical point of view that earlier life stages are more susceptible and sensitive to chemicals in general and EDCs in particular, the issue has not been adequately studied to support or reject this assumption. The few available studies provide conflicting results. Dregolskaya (1993) investigated the effects of chemical exposure on early life stages in *L. stagnalis* and the prosobranch snail *Bithynia tentaculata*. In both molluscs, the most vulnerable development stage was the last one before metamorphosis, when the protonephridium was reduced and a definitive kidney had not yet been built up. Bauer et al. (1997) have shown that the ability of TBT to induce intersex in the periwinkle *Littorina littorea* decreases with increasing age of the test organisms. Even exposure to very high TBT concentrations will not result in intersex development once female snails have reached sexual maturity. Also for a number of imposex-affected neogastropods like *Nucella lapillus* and *Ocenebra erinacea* a decreasing TBT sensitivity has been described (for review: Bryan and Gibbs, 1991) but in contrast to *Littorina,* adult and sexually mature females also preserve their ability to develop male sex organs under

TBT exposure. Other studies, as discussed in section 1.21.2 with the examples of Czech et al. (2001) and Leung et al. (2007) for effects of TBT in *L. stagnalis* and Oehlmann et al. (2000) for effects of bisphenol A and octylphenol in *M. cornuarietis,* found little evidence for a higher sensitivity of earlier life stages in FLC tests.

The knowledge about susceptible life history periods for EDC-induced effects in molluscs is too fragmentary to come to any final conclusions. However, it is worth mentioning that all reports on chemically-induced sex change in molluscs are based on experiments with an exposure of animals from early life stages (embryonic, larval or juvenile) through adulthood (Gibbs et al., 1988; Nice et al., 2003; Langston et al., 2007). This shows that developmental timing of exposure is critical and important at least if sexual differentiation is considered as a test endpoint. Although this observation reinforces the view that PLC tests may run the risk of not exposing the most sensitive life stage it has also to be emphasised that in the PLC test candidate species, *P. antipodarum*, females occur exclusively and the ovoviviparous mode of reproduction may probably reduce the possibilities of exposing early life stages during development in the maternal organism.

## 1.17 Routes of dosing

### 1.17.1 Water

Exposure via the water phase is the most common route of dosing in EDC studies with molluscs. In section 0 suitable exposure media for the candidate test species are proposed. Exposure or effect concentrations in the water phase from such tests can be directly correlated with the exposure level of field populations for risk assessment purposes, particularly because concentrations of chemicals can more easily be measured in the water and thus the database for aqueous concentrations is broader compared to those for concentrations in sediments or biota.

Application techniques for water exposures are well developed and established in many laboratories worldwide, either as semi-static (= renewal) or as flow-through systems. Because it is difficult to maintain a constant exposure concentration for readily biodegradable substances in semi-static experiments, the use of flow-through systems may be advantageous. The main drawback of flow-through testing is the higher instrumental effort, the increased risk of technical failure especially during experimental periods of several weeks or even months, the higher cost of chemical supply and contaminated water recycling, and the higher probability that nonpolar test compounds in particular can be adsorbed by tubing and pump material. Therefore, a clear steer as to whether a flow-through design is the more appropriate for EDC testing with molluscs cannot be given. A decision should be reached mainly depending on the stability of the test compound: semi-static systems are advantageous if the compound is sufficiently stable during the renewal cycles of exposure media, otherwise a flow-through design should be envisaged if the renewal time cannot be further reduced. Analytical measurements of exposure concentrations in the tanks have to be conducted at regular intervals independent of the chosen exposure system (cf. section 6.4).

Although the use of organic solvents should be avoided, it might be necessary to solubilize the test substance in the water and maintain it in solution, especially if nonpolar, poorly water-soluble substances are tested. This requires the inclusion of an additional solvent control in the test design. Solvents have to be chosen by the chemical properties of the substance. Recommended solvents according to OECD (2000) include acetone, dimethyl sulfoxide (DMSO), ethanol, methanol, tertiary-butyl alcohol, acetonitrile, dimethyl-formamide and triethylene glycol. However, some of the solvents recommended by OECD are toxic to humans (e.g. dimethyl-formamide, a possible carcinogen and teratogen; acetonitrile; teriary-butyl alcohol; methanol). To protect laboratory technicians these solvents should only be used if there is no less toxic alternative available and with technical measures to reduce the exposure of lab personnel (e.g. localised extraction of air above tanks). A further aspect to be considered is that these solvents were mainly recommended on the basis of short term toxicity tests with species other than molluscs so that further

investigations will have to address the question of their toxicity to the candidate test species, including potential endocrine disrupting properties.

For obvious reasons an ideal organic solvent should not be toxic to the test organisms and not readily biodegradable, limiting its use as a carbon source for microorganisms and consequent bacterial growth in the test system, which would increase maintenance time during the exposure and may favour the degradation of the test substance. According to OECD (2000), the maximum allowable concentration for solvents is $1/10^{th}$ of the NOEC or alternatively 100 mg/L (respectively 100 µL/L) if no toxicity data are available. While the OECD recommendation of a maximum solvent concentrations of 100 mg/L is supported by historical data, Hutchinson et al. (2006) report that some solvents may affect the reproduction of certain fish species, and also impact biomarkers of endocrine disruption. The authors recommend that maximum effort should be given to avoiding the use of carrier solvents wherever possible (e.g. by using saturation columns or other physical methods like stirring or ultrasonification). Where solvent use is necessary, however, they recommend that in reproduction studies with aquatic organisms, the maximum solvent concentration should not exceed 20 µL/L in the exposure medium.

The physico-chemical properties and aquatic toxicity of a range of solvents have also been reviewed by ECETOC (1996) and Rufli et al. (1998). There are no studies available which investigate the toxicity of solvents in molluscs systematically, however, in most of the published studies (reviewed in Lagadic et al., 2007 and Oehlmann et al., 2007 for pulmonate and prosobranch snails, respectively) ethanol has been used. In long-term experiments ethanol and other solvents such as acetone and methanol can be problematic due to substantial growth of bacteria in test vessels and tubing with resulting impact on the health status of the test organisms and the stability of the test compound. DMSO has been used by Schirling et al. (2006) in experiments with *Marisa cornuarietis* embryos and also in a number of yet unpublished experiments with *Potamopyrgus antipodarum* at the University of Frankfurt. This solvent had no effect on the tested endpoints at a concentration of 100 µL/L and did not cause growth of bacteria or biofilms in the test vessels.

Problems may also arise in testing of volatile, coloured or photolabile substances. Helpful guidance on testing of such substances is provided by OECD (2000) and ECETOC (2003).

### 1.17.2 Sediments

Many molluscs, including the species for which candidate test protocols are proposed in section 0, are benthic organisms living in close contact with the sediment. Sediments may act as both a sink for chemicals through sorption of contaminants to particulate matter, and a source of chemicals through resuspension. Sediments integrate the effects of surface water contamination over time and space, and may thus present a hazard to aquatic communities (both pelagic and benthic) which is not directly predictable from concentrations in the water column (EC, 2003). Especially for substances that are potentially capable of depositing on or sorbing to sediments to a significant extent, the toxicity to sediment-dwelling organisms has to be assessed. To avoid extensive testing of chemicals EC (2003) proposes a trigger value of log $K_{OC}$ or log $K_{OW}$ of $\geq 3$ for the conduct of sediment tests for sediment effects assessment.

Currently, there are limited toxicity data available for sediment dwelling organisms so that for risk assessment purposes the equilibrium partitioning method is often used to calculate the exposure to and hazard from chemicals in benthic organisms based on concentrations in the water phase (OECD, 1992). This approach suffers from various limitations and EC (2003) concludes that 'only whole-sediment tests using benthic organisms are suitable for a realistic risk assessment of the sediment compartment' because it is only possible by using such tests to adequately address all routes of exposure. Annex VI of EC (2003) summarises 8 sediment toxicity tests with 2 oligochaete, 2 insect, 3 amphipod and 1 nematode species with finalised international guidelines or guideline initiatives under development. One of the mollusc species proposed in section 0, the mudsnail *Potamopyrgus antipodarum*, has already been used for sediment tests (e.g. Duft et al., 2003a-c; Oetken et al., 2005; Mazurova et al., 2008; Schmitt et al. 2008) and a standard

operating procedure for this purpose is available (Duft et al., 2007).

Artificial sediment (cf. section 1.22 for details) may be preferable because uncontaminated natural sediments may not be available in required quantities and test results may be influenced by indigenous organisms as well as micropollutants. However, the type of sediment chosen should be based on a thorough understanding of appropriate husbandry parameters and life-history traits for the species.

Spiked sediments of the chosen concentration should be prepared by addition of a solution of the test substance directly to the sediment ('spiking'). A stock solution of the test substance dissolved in deionised water is mixed with the formulated sediment by rolling mill, feed mixer or hand mixing. If poorly soluble in water, the test substance can be dissolved:

- either in as small a volume as possible of a suitable organic solvent (e.g. ethanol, acetone or ethyl acetate). This solution is then mixed with 10 g of fine quartz sand for one test vessel. After evaporation of the solvent the sand is mixed with the suitable amount of sediment per test beaker.
- or alternatively in a larger volume of solvent (300 mL/kg sediment dw) to allow a complete soaking of the sediment and an equal distribution of the test compound in the sediment.

In either case the solvent has to be evaporated before the sediment is covered with water and the test is started. Furthermore, a solvent control has to be included in the experimental design if an organic solvent was used. However, note that in sediment toxicity tests of this type with a duration of up to 4 weeks, regular replacement of the spiked sediment should not be necessary to ensure constant exposure levels, except when testing readily biodegradable compounds.

### 1.18 Dose selection

For both PLC and FLC tests at least 5 concentrations with four replicates should be tested in a geometric series with a spacing factor between concentrations not exceeding 2.2 to provide sufficient statistical power (cf. section 6.5.3) and an adequate calculation of effect concentrations (cf. section 6.5.5). In the case of a non-monotonic concentration-response relationship (e.g. low dose effects or hormesis, cf. section 1.32) the consideration of additional treatments in the lower concentration range with a smaller spacing factor may be advisable. Furthermore a (negative) control, a positive control (e.g. 25 ng EE2/L for *Potamopyrgus antipodarum* when testing a suspected estrogen) and a solvent control (if appropriate) have to be considered for the test. Prior knowledge on the toxicity of the test substance (e.g. from range finding studies) should help in selecting appropriate test concentrations. For a range finding experiment three widely spaced treatment levels separated by up to an order of magnitude would be appropriate. The highest concentration for the definitive test should be less than lethal and less than the water solubility of the test compound. At least one of the test concentrations should be below the predicted no-observed-effect level.

If the stability of the substance in the test system has been established, exposure concentrations should be measured at regular intervals, depending on the exposure design. In semi-static (= renewal) systems where the concentration of the test substance is expected to remain within ± 20% of the nominal (i.e. within the range 80-120%), it is recommended that, as a minimum, the highest and lowest test concentrations are analysed in samples from the same solution when freshly prepared and immediately before renewal (cf. OECD, 1998) in the first and last week of a PLC test and at 2 weeks-intervals in FLC tests. For tests where the concentration of the test substance is not expected to remain within ± 20% of the nominal, it is necessary to analyse all test concentrations, when freshly prepared and at renewal. In all cases, determination of test substance concentrations prior to renewal has to be performed on at least one replicate vessel at each test concentration. For flow-through systems the measurement of 'old' solutions is not applicable. Instead it is advisable to increase the number of sampling occasions during the first week (e.g. three sets of measurements), followed by a further sample per treatment at weekly intervals thereafter. In these types of test, the flow-rate of water and test substance should be checked daily. Analytical control of exposure levels is also necessary for sediment tests. Like in PLC tests with exposure via water, as a minimum, the highest and lowest test concentrations should be analysed in the first and last week of the

experiment.

If the concentration of the substance being tested has been satisfactorily maintained within ± 20% of the nominal or measured initial concentration throughout the test, then results can be based on nominal values. If the deviation is greater than ± 20%, results should be expressed in terms of the time-weighted mean (cf. annex 6 in OECD, 1998).

When planning the test, it should be taken into consideration whether the aim is to determine the NOEC/LOEC (by use of ANOVA or comparable nonparametric tests) or $EC_X$ values (by use of (non )linear regression) (cf. section 1.19.5).

## 1.19 Statistical considerations

The objective of the PLC and FLC tests with molluscs is to provide the most ecologically-relevant, economic and statistically efficient estimate of toxicity for a given chemical, including apical endpoints under endocrine control. The selected tests and endpoints must be biologically sensitive with an acceptable exposure-associated variability during the test, and should be statistically powerful. The biological sensitivity is a function of the selected test species and analysed endpoints, and of the route, level and duration of exposure. It is furthermore influenced by the ecological relevance of the endpoints measured, i.e. their suitability to extrapolate effects from individuals to the community level and from the laboratory to the field condition. The sensitivity of the test is negatively correlated with its inherent variability (i.e. intraspecific variability between individuals) and design-associated variability (i.e. dosing of chemicals, exposure route and duration, chemical stability and purity within the testing environment, testing protocol, light and temperature heterogeneity between replicates).

Extensive discussion of the statistical analysis of ecotoxicity data is provided in OECD (2006).

### 1.19.1 Controlling variability

It is important to recognize that molluscs are characterised by a high inherent variability for many of the potential endpoints to be considered in PLC and FLC tests, including reproductive and developmental parameters. In particular, reproductive output (i.e. numbers of clutches and eggs or embryos produced) as an important endpoint of fecundity, varies considerably between species and even between individuals of the same species. For *Potamopyrgus antipodarum* and *Lymnaea stagnalis*, the reproductive output depends on shell height so that size classes have to be defined for the use in the tests (cf. sections 7.3 and 7.4.1). It is, however, also important to notice that this inherent variability is a natural trait of almost all systematic groups in the animal kingdom, not limited to molluscs, and thus not a disturbing factor *per se* although it clearly affects the statistical power when analysing ecotoxicity data. This characteristic has to be taken into account rather than attempting to reduce the natural variability by choosing test species with less inherent variability, although such data are only available for very few species. Although the latter strategy might be advantageous at first glance it is questionable whether such species are representative of their taxonomic group. In particular, it is considered important to measure reproduction and development in several randomly selected groups and to critically evaluate the question of defining the experimental unit in order to minimise the effects of inherent variability on statistical power.

The design-associated variability is reduced by minimising the variability of the chemical purity of the test compound and of exposure levels (i.e. test concentrations) through the duration of the test. Both aspects can be controlled by appropriate chemical analyses of exposure media (water and sediments, cf. section 1.18). The use of test species with shorter life cycles may reduce the required time for PLC and FLC studies and by default reduces the design-associated variability in the exposure. However, it has to be considered that such r-strategists are usually characterised by a higher inter-individual variability because their life strategy allows them to deal with rapid changes of environmental conditions by responding to suitable conditions with a higher rate of reproduction (Schäfers and Nagel, 1991; Raimondo et al., 2006).

Again, the need for a high statistical power of the test and factors influencing its ecological relevance are conflicting, so that a reasonable balance between statistical and ecological requirements has to be found.

The experimental design should consider randomness, independence, and replication (Cochran and Cox, 1992). Randomness and independence are used to remove bias, and replication provides a measure of variability across similar test units within a treatment (Chapman et al., 1996a). Ideally, all three factors should be maximised from a statistical point of view, however, this is usually not possible for practical reasons particularly regarding the number of replicates, so that an adequate test design is always a compromise between statistical requirements and practicability.

### 1.19.2 Defining the experimental unit

The experimental unit is defined as the group of material (i.e. organisms in the present case) to which a treatment is applied independently in a single trial of the experiment (Cochran & Cox, 1992). The replication of experimental units for each treatment provides a measure of all potential sources of variability needed to extend the inference across time and space (OECD, 2008). Pseudoreplication is defined as the use of inferential statistics to test for treatment effects with data from experiments in which either treatments are not replicated (though samples may be) or replicates are not statistically independent. For either type of pseudoreplication an error term is obtained that is invalid for testing the hypothesis associated with the mean responses from the group of animals tested over time and space. Without true treatment replication, the effect of the treatment is confounded with the variability of the response (OECD, 2008). However, true replication in standard ecotoxicity testing is rarely achieved. If, for example, only one solution for each treatment is made and then divided between replicates, the source of variation associated with the formulation of the treatment is not considered in the variability for testing. Thus no true replicates have been established from a statistical point of view although it can be argued that this factor is too small to be of concern and too costly to include.

For most endpoints in PLC and FLC test with molluscs, such as survival, sex ratio, growth (e.g. shell height or weight), development (e.g. hatching success, time to hatch or first reproduction) and fecundity (e.g. eggs, clutches, eggs/clutch and released offspring), the experimental unit is the tank. In these cases a relatively large number of replicate tanks is needed for sufficient power to detect significant differences between treatments and the control. However, for endpoints based on individual measurements, particularly for the number of embryos in the brood pouch of *Potamopyrgus antipodarum*, it can be argued that the individual female is the experimental unit (see also extensive discussion in OECD, 2008). Although some statisticians argue that this approach ignores pseudoreplication (Hurlbert, 1984), others emphasise that it may not be practically possible to run ecotoxicity tests for such endpoints without treating individual animals as individual replicates even if they are housed together. But even in these cases a minimum number of tanks per treatment – at least two and preferably four – has to be considered in the test design. Treating the individual as a replicate makes it necessary to provide information on within-tank and between-tank variability. This can be achieved using a nested ANOVA or by indicating statistically significant differences between tanks of a given treatment if all individuals of this treatment are analysed for treatment effects by using ANOVA or comparable nonparametric tests (cf. section 1.19.5).

An alternative approach, the weighted mean method (Taylor 1982), is based on common error propagation rules and considers the tank as the experimental unit, but takes into account the within-tank (i.e. individual animal) variability: the mean animal performance per tank and the standard error of the mean (SEM = SD/sqrt[n]) are calculated. From these the weighted mean and SEM are calculated per treatment group (n = number of tanks per treatment). These weighted means (with SEM and n) are analysed for treatment effects using ANOVA or non-parametric tests (cf. section 6.5.5). By using this method the individual variation is not lost, but explicitely taken into account in the statistical analysis of the results if for logistical reasons high replicate numbers cannot be achieved.

### 1.19.3 Importance of statistical power

The power of a statistical test is the probability that the test will reject a false null hypothesis (that it will not make a Type II or β error). Power analysis can either be done before (*a priori*) or after (*post hoc*) data collection in an experiment. The *a priori* analysis intends to determine the adequate number of experimental units to achieve the desired power for identifying differences between a treatment group and the control. *Post hoc* power analysis is conducted after a study has been completed, and uses the actual sample size and effect size to determine what the power was in the study. A free statistical power calculator is provided on the internet under http://www.dssresearch.com/toolkit/spcalc/power.asp. Although there are no formal standards for power, most researchers who assess the power of their tests use values in the range between 60% and 80% as the lower threshold for adequacy.

The statistical power of a given test design decreases (and thus the chance of a Type II error increases) with the variability between replicate experimental units (i.e. within a treatment) and increases with the number of replicate experimental units, the size of the Type I or α error (i.e. the significance level that is chosen, usually 5% (p = 0.05)), and the percentage of difference to be detected. Only the latter three parameters can be controlled in a test while the response variability is inherent to the test organism. Thus, a characterisation of the natural or inherent variability (e.g. as coefficient of variation, CV) of the analysed endpoints in a given test species is required for power calculations (Figure 6-1). Endpoints with high CVs as a result of considerable inherent variability have a low power for detecting small-scale differences. This is exemplified in Figure 6-1 for control fecundity data in an experiment with *Lymnaea stagnalis* with 2 replicates per treatment. The measured response with a CV ≥ 15% will be unlikely to detect differences smaller than 50% between the treatment and reference response at a significance level of p = 0.05 if a statistical power of at least 60% has to be achieved. For a given CV the power can be increased by increasing the number of replicates.

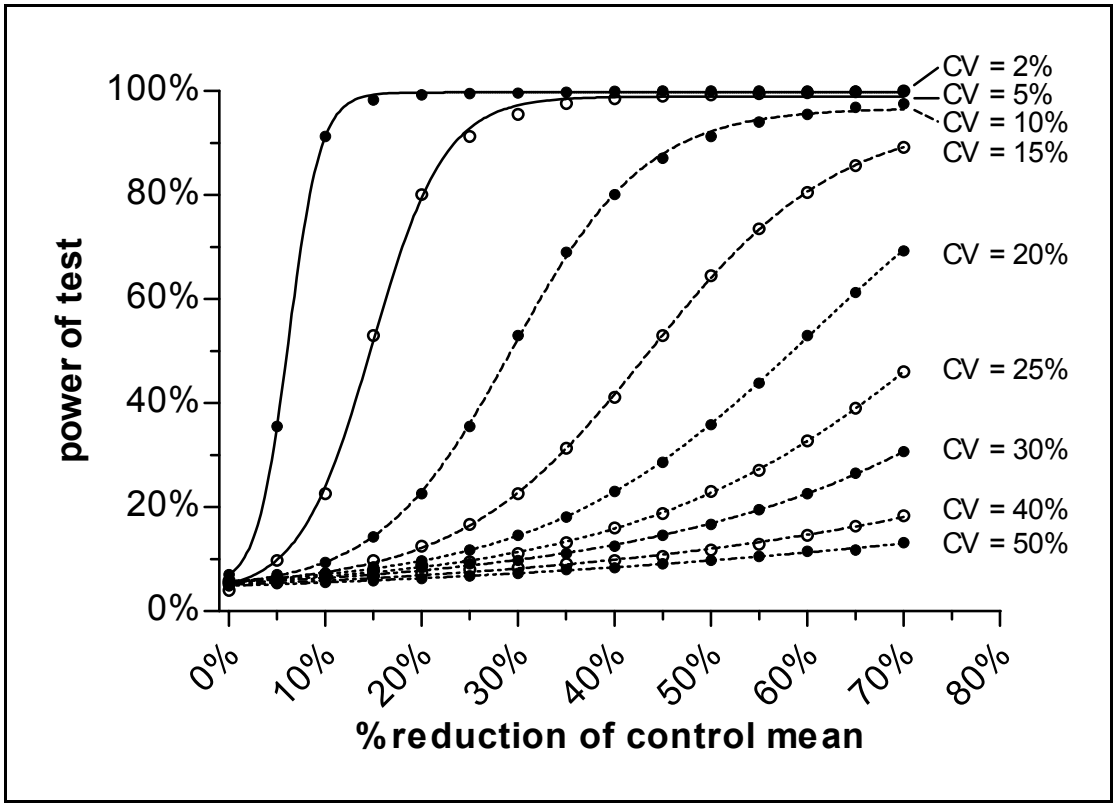

Figure 0-1: Power of a one-sided *t*-test for independent samples as a function of the percent reduction detected between the test and reference means with 2 replicates per treatment (p = 0.05) ( modified from OECD, 2008).

### 1.19.4 Data treatment

Parametric tests (e.g. *t*-test or ANOVA) are preferred for data analysis because they have a greater power of discrimination compared to non-parametric tests (OECD, 2006). However, parametric tests are based on assumptions that the responses being analysed follow an approximately normal distribution and exhibit equal variances. The normality assumption can be met for quantal data (e.g. survival or mortality, proportion of females or males) only if the experimental design includes replicates for all treatment groups. Only in cases that data do not meet the requirements for parametric tests (possibly after a transformation), should non-parametric tests be applied (e.g. Mann-Whitney, Wilcoxon, Kruskal-Wallis or Friedman test). Before using a non-parametric test, the following data transformations should be applied to establish normal distribution and homogeneity of the within treatment variances as recommended by OECD (2006). If data already meet the requirements for parametric tests, then no transform is needed:

- quantal responses expressed as a percentage (e.g. survival or mortality, proportion of females or males) will be arcsine-square root transformed,
- counts (e.g. numbers of produced eggs and clutches or number of embryos in the brood pouch) should be square root transformed,
- continuous data (e.g. growth or weight measures) should be transformed to the natural logarithm.

Tests for the comparison of two samples (e.g. *t*-test, Mann-Whitney and Wilcoxon for counts and continuous data; Fisher's exact test for quantal and binomial data) can be used to compare two test vessels and check whether there is any statistically significant difference (e.g. between water control and solvent control, when a solvent carrier was used in the test) before deciding to pool them for the increase of statistical power in the further statistical analysis (OECD, 2006).

Statistical analyses can be conducted both with and without suspected outliers (Chapman et al., 1996a). Outliers can be identified by three approaches:

- Values that exceed the median plus 3 times the inter-quartile range (i.e., the difference between the 1$^{st}$ and 3$^{rd}$ quartiles).
- Tukey (1995) suggests defining an outlier as an observation more than 1.5 times the inter-quartile range above the 3$^{rd}$ quartile or 1.5 times the inter-quartile range below the 1$^{st}$ quartile. This rule is implemented in standard software packages such as SAS and identifies a higher percentage of observations as outliers than the first cited method.
- Application of outlier tests like the Grubbs Test, which is also called the extreme studentised deviate (ESD) method (Grubbs, 1950).

If there are no changes to the results, then the analysis including the outliers should be presented. If differences occur, then the implications of removing the outliers should be carefully documented. If an explanation can be made as to why there are outliers, the analysis excluding outliers may be sufficient.

### 1.19.5 NOEC and $EC_X$ determination

The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) have widely been used to evaluate data from chronic ecotoxicity tests, including PLC and FLC studies (OECD, 2006). There has been a long debate over the use of the NOEC in toxicity assessment and the associated risk analysis (Chapman et al., 1996a & b; Crane and Newman, 2000). The calculation of the NOEC is based on the comparison of means, e.g. by ANOVA or comparable nonparametric tests followed by appropriate post tests (cf. below and Figure 6-2). This approach is appropriate to compare means (e.g. are treatment means statistically different from the control?) in hypothesis testing (e.g. does the test chemical

affect reproduction at a given concentration?). However, this approach is not appropriate when accurate estimates of toxicity and precise patterns of responses are required. Regression techniques provide an estimate of the level of effect as a function of exposure and the functional relationship between nominal or actual concentration and response. This also allows a comparative assessment of sensitivities and of potential thresholds of effects of different endpoints by analysing the concentration-response relationships.

Even though the NOEC is widely used, it should not be relied on as the sole indicator of low or negligible toxicity. The highest test concentration for which statistical differences have failed to be detected is a direct function of the power of the test and of the choice of test concentrations. For certain endpoints, such as fecundity parameters in egg-laying species such as *Lymnaea stagnalis*, it is unlikely that any effects will be detected with only two replicates per treatment, given the variability between replicates.

Statistical tests for NOEC/LOEC determination

A decision tree for an appropriate statistical analysis to determine the NOEC and LOEC in PLC and FLC tests with molluscs is given in Figure 6-2. For all endpoints where the tank is the experimental unit, mean values per tank are considered for the subsequent statistical analysis. Endpoints based on individual measurements (e.g. the number of embryos in the brood pouch of *Potamopyrgus antipodarum*) should be analysed using error propagation rules as outlined in section 6.5.2: Weighted means and SEMs are calculated per treatment group (with n = number of tanks per treatment) and taken for the subsequent statistical analysis.

Regression analysis for ECx determination

An alternative to the NOEC determination is the analysis of regression to determine a given effect concentration $EC_X$, if an acceptable regression model fit to the data can be found. However, care must be taken not to estimate an $EC_X$ value that lies outside the range of the data (i.e. use interpolation and not extrapolation) because this introduces large error limits (wide confidence intervals). The difficulty in interpreting the results from PLC and FLC studies with molluscs is to decide whether a statistically significant or non-significant result will have population-level effects i.e. what size of $EC_X$ is considered biologically significant, although this is also a problem when interpreting NOEC and LOEC values.

The design and analysis requirements differ substantially for studies which aim to calculate the NOEC or $EC_X$ (Chapman et al., 1996a; OECD, 2006). The calculation of the NOEC requires experimental unit replication and achieves greater power in testing as a function of the number of replicates (cf. section 1.19.3). In contrast, the calculation of an $EC_X$ requires a higher number of treatments (test concentrations) but not necessarily replication within the treatments. While the main drawback of the NOEC approach is the complete ignorance of the concentration-response nature of the study, the objection to the regression approach is the use of specific regression models to capture this concentration-response when there is no agreed biological basis for any routinely used model.

Benefits of the regression approach include 1) estimation of the pattern (e.g. slope) of toxicity as a function of concentration; 2) estimation of the distance between effect concentrations and environmental concentrations; 3) estimation of $EC_X$ and the associated confidence intervals for x equal to a low to medium effect; 4) the $EC_X$ estimates are not limited to tested concentrations; and 5) the ability to compare concentration-response curves across endpoints (Chapman et al., 1996a; OECD, 2008). The size of the resulting confidence intervals and thus the precision of the estimated $EC_X$ is a function of the inherent variability in the response and the number and spacing of the concentrations tested.

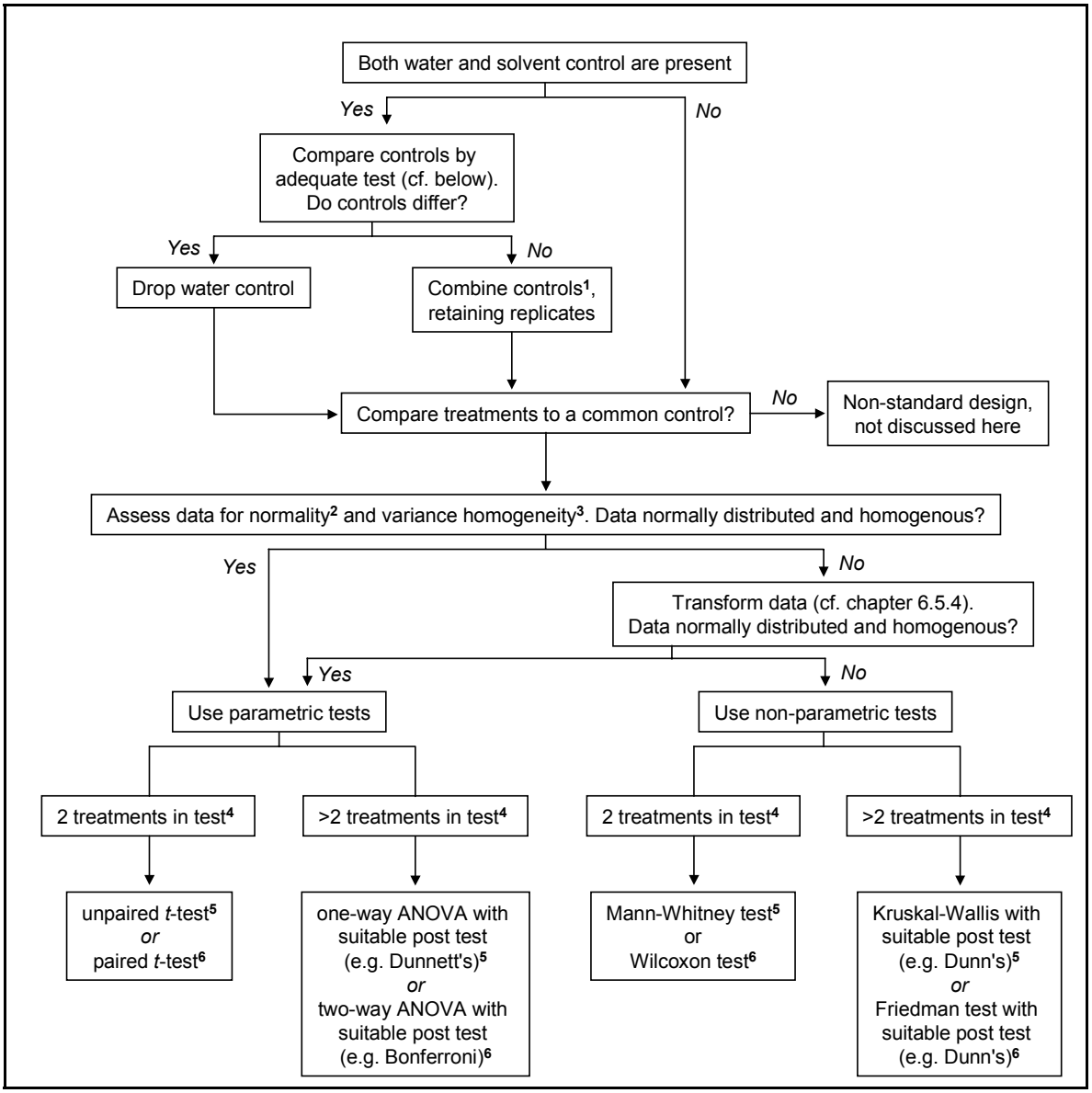


Figure 6-2: Statistical flow chart describing the recommended statistical protocol for NOEC/LOEC determination in mollusc PLC and FLC tests with ≥ 5 experimental units per treatment (modified from OECD, 2008).

[1] It should be noted that scientific judgement and regulatory guidance must be considered in deciding whether to pool water and solvent control.

[2] e.g. by using the D'Agostino-Pearson, Shapiro-Wilks, Anderson-Darling or Kolmogorov-Smirnov test;

[3] e.g. by using Levene's or Bartlett test; [4] including zero treatment (= control);

[5] for unpaired observations; [6] for paired or blocked observations.

Most OECD guidelines for chronic tests require at least 5 concentrations that are geometrically spaced and sublethal, plus a control. Thus, a range-finding test would be required to determine appropriate concentrations. Regression modelling is flexible enough to handle a wide range of concentration-response patterns including non-monotonic. Furthermore, it can handle a wide range of responses including continuous responses, counts, and quantal data by re-expressing or transforming the data (e.g. log (y+c);

$(y+c)^{1/2}$, and probit respectively).

In summary and in agreement with the conclusions reached by OECD (2008) for fish tests, ANOVA designs for testing with molluscs appear inferior to regression designs. The latter are considered to show more promise for mollusc PLC and FLC tests given the generally large inherent variability in fecundity and developmental parameters between individuals, which inevitably reduces the power of the ANOVA approach and are therefore the recommended design.

### 1.19.6 Sample size in FLC tests

For the planning of FLC studies with molluscs it has to be ensured that the sample size is sufficient to produce an adequate number of offspring for analyses in the succeeding F1 generation. If reproduction fails at high concentrations or the number of surviving offspring is low due to high lethality of early life stages, the assay approximates a screening test, as only F0 reproductive effects can be assessed. To guard against this possibility, fertilisation and hatching success should be measured in a screening test and carefully scrutinized to avoid excessively high exposures. Furthermore, it is advisable to increase the number of adults in F0 replicates at high exposure levels to ensure adequate collection of surviving F1 specimens.

For FLC studies with fish it has been recommended to start the F0 exposure with 100 embryos per replicate (OECD, 2008). This number also seems reasonable for FLC studies with molluscs and ensures adequate numbers of early life stages can be procured for continuation of the exposure to maturity. A sample size of 100 allows for a good examination of survival, development and growth of the F0 generation. It also allows for adequate numbers of adults to set up reproduction groups and collect sexually mature specimens for biomarker and histopathological analyses. Details of recommended replicate numbers which determine the statistical power of the test are provided in section 7.3 and 7.4.

**Candidate test protocols**

As recently reviewed by Matthiessen (2008), no nationally or internationally standardized methods for a test providing effect data derived from endocrine-mediated mechanisms with molluscs are available. Although considerable efforts have been made by several organisations including the International Organisation for Standardisation (ISO), the International Council for the Exploration of the Sea (ICES), the American Society for Testing and Materials (ASTM) and the US Environmental Protection Agency (USEPA) to implement test protocols (ISO, 2006; ASTM 2007 a & b; Chapman et al., 1995; Gibbs, 1999; Oehlmann, 2004; Thain, 1991; Widdows and Staff, 2006) the resulting methods are of limited applicability for the study of EDCs. Some of them are acute tests, others bioassays for quality assessment of marine waters or contaminated soils, whilst others focus on biomonitoring studies with animal samples from the field.
Even though some of the biological processes measured by these methods are under hormonal control, it is doubtful whether they can be modified for EDC testing as they only cover a small part of the life cycle and/or do not provide a measure of reproductive success. Notwithstanding the usefulness of these guidelines, the current state of knowledge makes it advisable also to examine other published methods.

### 1.20 Strengths and weaknesses of potential test species

It will be apparent from section 0 that there are many culture and test design criteria which need to be considered when choosing mollusc species for potential test development and validation. Using these criteria, Appendix 1 presents the information available on a range of mollusc species, obtained both from the literature and by personal communications with leading scientists in this field. This information has been linked to the criteria proposed by Gourmelon and Ahtiainen (2007) and Hutchinson (2007) concerning invertebrate test methods for regulatory assessment of chemicals and is condensed in Tabel 7-1 into broad categories considered to be the most important for identifying suitable species for further examination. Data on 21 species have been gathered in these tables, representing 5 bivalves (1 freshwater and 4 estuarine/marine), 9 marine/estuarine gastropods, and 7 freshwater gastropods. Although other mollusc species have been used as partial- or full-life cycle test organisms from time to time, the available data from these is too sparse to be useful. The data in Appendix 1 are not complete in all cases (and it is possible that a few important species have been inadvertently omitted), but sufficient information is available to allow the field of potential test species to be narrowed down to a smaller sub-set worthy of closer examination.
The list of possible test species is not intended to be prescriptive, and research would be desirable to suggest additional candidates. However, it is considered that an over-riding criterion to be used in the context of this review is whether or not a species can be easily induced to breed in the laboratory. This is partly because wild-caught animals will have heterogeneous genetic backgrounds, and unknown pollution exposure histories, but also because many molluscs are susceptible to parasitic infections, some of which can cause conditions that mimic endocrine disruption, or otherwise influence the molluscan endocrine system (Morley 2006, 2008). It is therefore essential to be able to set up breeding colonies which are free of both contaminants and parasites. Simply being able to culture captured molluscs in the laboratory is insufficient because of the problems with wild-caught material.
Eliminating molluscs that are currently hard or impossible to breed in the laboratory narrows the current field to just 7 or 8 species, including the marine bivalves *Crassostrea gigas* (and possibly *Crassostrea virginica*) and *Ostrea edulis*, the marine gastropod *Crepidula fornicata*, and the freshwater gastropods *Potamopyrgus antipodarum*, *Marisa cornuarietis*, *Lymnaea stagnalis*, and *Planorbarius corneus*. It is

possible that further research will develop laboratory breeding methods for otherwise suitable testing species, but this list is considered to represent the species which are currently amenable to routine laboratory production and potentially suitable for apical life cycle testing of EDCs and other chemicals.
Considering the bivalves first, the European flat oyster *Ostrea edulis* and the Pacific oyster *Crassostrea gigas* are both widely available due to their use as human food and their ability to thrive in mariculture systems. *Crassostrea virginica* is also grown in mariculture, and all 3 species have been used to a greater or lesser extent in ecotoxicology. All are marine, with planktonic larval stages, and they filter-feed on micro-algae. Although methods for breeding and culturing are well-known, they are expensive, space-hungry and time consuming, requiring abundant supplies of clean seawater and the ability to grow several species of marine micro-algae as a food supply (although preserved algal foods for use in the aquaculture industry are now available). However, whereas experience in using *O. edulis* for testing the effects of EDCs seems to be limited to a single study with tributyltin (Thain and Waldock, 1986), there is more experience with *C. gigas*. In particular, it appears to be sensitive to triorganotins (Thain and Waldock, 1985; Le Curieux-Belfond *et al*., 2001), estradiol (Li *et al*., 1998), and alkylphenols (Nice *et al*., 2003). Furthermore, it should also be noted that life cycle experiments have been successfully conducted with *C. gigas* (Nice *et al*., 2003), in which freshly-fertilised embryos were exposed for 48 h to nonylphenol at 1 µg/l, and then allowed to reach adulthood in clean water in a hatchery, whereupon the eggs were stripped and fertilised *in vitro*, and allowed to develop to the D-larval stage. This treatment not only induced hermaphrodites in the F0 adults, but also reduced the production of F1 larvae. There is less experience of life cycle tests with *C. gigas* than with *L. stagnalis* (see below), and they take longer (10 months compared with 6 months), but they appear to be practical and sensitive to some EDCs.

Given the relative lack of experience with marine species and large-scale algal culturing in most chemical testing laboratories, and the tendency of aquatic environmental risk assessments to focus on freshwater species, there may be a lack of demand for tests based on *C. gigas* or similar bivalves. However, in recognition of possible future (though probably limited) requirements for partial- or full life cycle testing based on bivalves, this species shows sufficient promise to merit investigation of potential test protocols.
The only one of the marine gastropods listed in Table 7-1 which can be bred fairly easily in the laboratory (Calabrese and Rhodes, 1974) is the mesogastropod slipper limpet *Crepidula fornicata*. This filter-feeding species (which like *C. gigas* requires abundant seawater and cultured micro-algae) is widespread in North America, and has been introduced to much of Europe. It is a protandrous hermaphrodite which lives in stacks of several individuals where young males attach to larger females, and gradually change into females themselves. Eggs are fertilised internally and the larvae are then released into the plankton. It can be exposed successfully to chemicals over long periods in the laboratory (Nelson *et al*., 1983), and there is some information (Thain, 1984) which shows that reproduction and subsequent larval survival in this species is impacted by low concentrations of mercury. However, cadmium does not produce such effects (Pechenik *et al*., 2001), and there is no information on the influence of other suspected EDCs. This species may nevertheless be a good one for studies of EDCs, given its sex-changing lifestyle which may be sensitive to chemical interference, but at the present state of knowledge it can only be recommended as a subject for future research.

The majority of laboratory-based research on the effects of EDCs in molluscs has been conducted with various freshwater gastropods. Perhaps the most promising species yet investigated for partial life cycle testing is the ovoviviparous mesogastropod *Potamopyrgus antipodarum* (synonyms: *Hydrobia jenkinsi* and *Potamopyrgus jenkinsi*), known as the freshwater or New Zealand mudsnail. It was introduced to Europe from New Zealand in the 19$^{th}$ Century and while mainly reproducing parthenogenetically, it has become the most common freshwater gastropod in several European countries (Wallace, 1985). European populations appear to be entirely modified triploid parthenogens (female only) with three distinct clonal lines (A, B, C) showing minor differences in life-history traits and sensitivities among clones (Jacobsen and Forbes 1997; Jensen and Forbes 2001). Although this may also have implications for the endocrine control of reproduction, the species exhibits a remarkable sensitivity to EDCs (cf. below). The species lives

in brackish- as well as freshwater (salinities up to approximately 15 ‰), so could be used for investigating the effects of contaminants present in either medium. In the laboratory, it can produce up to 4 generations per year, and under optimum conditions it can reach sexual maturity quickly (3 months) although there is some disagreement in the literature (Soares, 1992; Jensen *et al*., 2001; Pedersen *et al*., 2009). It is relatively cheap and quick to culture and test (either in water or sediment), and requires little laboratory space (Duft *et al*. 2003a & b, 2007). Embryo production in this species responds to a range of EDCs, including triorganotins (Duft *et al*., 2003a), ethinylestradiol (EE2), nonylphenol (NP), octylphenol (OP) and bisphenol A (BPA) (Duft *et al*., 2003b; Jobling *et al*., 2004), methyltestosterone (MT) (Duft *et al*., 2007), and the UV screens 3-benzylidene-camphor and 3-(4'-methylbenzylidene)-camphor (Schmitt *et al*., 2008). Although the sensitivity of *P. antipodarum* to some of these substances deviates from those of fish, the overall response is comparable (Jobling *et al*., 2004). Therefore, it certainly looks like a strong candidate for further test development, and indeed, the 28-56 d partial life cycle reproduction test method with this species has already been proposed for standardisation (Duft *et al*., 2007).

Another potentially useful species is the mesogastropod ramshorn snail *Marisa cornuarietis* which is native to tropical and sub-tropical regions of North and South America. This gonochoristic species lives primarily in freshwater, but also tolerates brackish water up to 8.5‰. It takes longer than *P. antipodarum* to reach sexual maturity (3-8 months, depending on conditions), but nevertheless can go through 3 generations per year. Culturing and testing costs are higher than for *P. antipodarum*, mainly due to longer test duration, but it is possible to run full life cycle tests (Meier-Brook and Tjhen, 1977; Schulte-Oehlmann *et al*., 1995; Oehlmann *et al*., 2006; Schirling *et al*., 2006). Depending on the EDC and test in question, sensitive endpoints include imposex, egg production, gonadal morphology, hatching success and embryo development. *M. cornuarietis* is responsive to several EDCs, including TBT and TPT (Schulte-Oehlmann *et al*., 1995, 2000), the anti-androgens CPA and vinclozolin (Tillmann *et al*., 2001), and the estrogens EE2, and BPA (Oehlmann *et al*., 2006; Schirling *et al*., 2006). However, in the case of BPA, observations of excess egg production (so-called super-feminisation) by Oehlmann *et al*. (2006) have not been replicated (Forbes *et al*., 2007a & b). This appears to be due to a variety of experimental design considerations, not the least of which is the apparent temperature-dependence of the super-feminisation response (although this observation is still the subject of controversy), which may only occur when the animals experience lowered temperatures. Furthermore, *Marisa* exhibits a number of different strains, some of them having an external sex dimorphism but no seasonal pattern in reproduction while in other strains males and females cannot be distinguished externally and reproduction is clearly seasonally with a two months spawning season at the end of the year. These substantial dissimilarities may even point to the existence of a species complex with at least two cryptic species as it has been shown for a number of other gastropods (Wilke et al 2002 and Pfenniger et al 2003, as cited in Oehlmann et al., 2008b). These controversial issues have not been fully resolved, but taken together with the higher culturing and testing costs of *M. cornuarietis*, it suggests that this species may not be as suitable as *P. antipodarum* for further test development and validation at this stage. However, this situation could change if optimum test conditions for this species were to be fully defined.

A promising candidate for possible use in partial and full life cycle tests with gastropods is the facultatively self-fertile hermaphrodite pulmonate *Lymnaea stagnalis*, known as the great pond snail, a holarctic species found throughout North America, Europe and northern Asia. *L. stagnalis* is one of the few molluscan species whose endocrinology is reasonably well understood (see section 0). It reaches sexual maturity in a similar time to *P. antipodarum* (3-4 months), and can go through 3 generations per year. It has low or medium culturing and testing costs, and EDC-sensitive endpoints include egg production, embryo development, hatching success, growth rate (Brown, 1979; Wilbrink *et al*., 1992; Czech *et al*., 2001; Segner *et al*., 2003; Weltje *et al*., 2003; Lagadic *et al*., 2005; Coutellec and Lagadic, 2006; Ducrot *et al*., 2008a, b) and a number of biomarkers including hormone titres (Jumel *et al*., 2002), metabolizing enzymes (Wilbrink *et al*., 1991; Teunissen *et al*., 1992), and immune responses (Russo and Lagadic, 2004; Russo *et al*., 2007, 2008). *L. stagnalis* is known to respond to several EDCs, including vinclozolin (Lagadic *et al*.,

2005, Weltje *et al*., 2003), CPA (Weltje *et al*., 2003), TBT (Czech *et al*., 2001; Leung *et al*., 2007), EE2 (Segner *et al*., 2003; Casey *et al*., 2005), NP (Lalah *et al*., 2007), β-sitosterol and MT (Czech *et al*., 2001), and the herbicide fomesafen (Jumel *et al*., 2002), although not always at environmentally-relevant concentrations. As with *P. antipodarum*, there seems to be enough experience with *L. stagnalis* to justify further investigation of both PLC and FLC test protocols.

The facultative self-fertilisation of *L. stagnalis* might be an important factor when choosing a test design; it implies that exposures of a single individual undergoing autogamic reproduction as well as exposures of several individuals performing sexual reproduction are possible. Exposure of multiple individuals improves statistical power. On the other hand autogamy may lead to a loss of genetic diversity. Furthermore, when testing EDCs the test design that allows sexual reproduction might be more appropriate since it is not known under what environmental conditions self-fertilisation is fostered and which (and how) hormones are involved in self-fertilisation compared to sexual reproduction.

Based on presently-available information, the final freshwater gastropod species with possible potential for reproductive test development and validation is another pulmonate, the great ramshorn snail *Planorbarius corneus*. This hermaphroditic species is widely distributed in small streams and ponds across Europe and central Asia. There is some experience in using it for reproduction experiments (e.g. Mazuran *et al*., 1999), but none involving EDCs have yet been published. However, research at Brunel University (Clarke *et al*., 2009) shows that exposure to estrogenic sewage effluent increases egg production in this species. Furthermore, longer-term exposure to various estrogens in the laboratory, as well as causing increased egg production in the F0 generation, leads to developmental mortality, weakened gametogenesis and poor reproduction in the F1 offspring, suggesting that enhanced egg production should not be seen as beneficial. Unpublished work (Dr. Ed Routledge pers. comm., 2008) shows that estradiol is able to increase egg production back to previous levels in *P. corneus* whose fecundity has been reduced by lowered autumn temperatures, while no change in reproductive output is seen at higher summer temperatures. This shows the importance of temperature control in experiments of this type (cf. research with *M. cornuarietis*). Culture and reproduction experiments with this species are of similar cost and duration to those involving *M. cornuarietis* (i.e. moderately high), but detailed operating protocols for work with *P. corneus* have not yet been published, and the limited data on sensitivity to EDCs prevents firm recommendations for development and validation of test procedures at this stage. This situation may change when more research on this species has been published. There has also been limited experience with the use of *Planorbis carinatus* for testing chronic effects of the pharmaceutical ibuprofen (Pounds *et al*., 2008), but again, more work would be needed before this species could be recommended for development of guidelines sensitive to EDCs.

In summary, there is good evidence concerning the suitability of the freshwater gastropods *Potamopyrgus antipodarum* and *Lymnaea stagnalis* for use in partial- and full- lifecycle tests of EDCs, respectively. An FLC test protocol with *P. antipodarum* and a PLC test with *L. stagnalis* could also be easily established. A candidate PLC protocol for *Lymnaea stagnalis* has indeed been developed and proposed by colleagues from INRA (Rennes, France) and is provided in Appendix 2 of this document. Although more expensive to culture and test, the bivalve *Crassostrea gigas* also shows promise for full life cycle testing, particularly if there is a regulatory need for data from bivalves or marine species. Of the other species discussed in this section, it is considered that further developmental research is required before *Marisa cornuarietis* or *Planorbarius corneus* could be recommended for test optimisation and validation, particularly concerning temperature-dependency of effects and taxonomic status (*M. cornuarietis*) and sensitivity to a wider range of EDCs (*P. corneus*). *Ostrea edulis* and *Crepidula fornicata* also have desirable properties, but almost nothing is known about their sensitivity to EDCs, so these also cannot be recommended for test optimisation at this time.

Table 7-1: Summary of the attributes of various potential test species, based on information in Appendix1.

| **Species** | **Easily bred in the lab** | **Cheap to conduct tests** | **FLC/MGS* available** | **Compact space require-ments** | **Rapid generation time** | **large number of offspring produced in the lab** | **Apical endpoints easy to assess** | **Known sensitivity to some EDCs**** | **Significant information on culture & lab testing available (in English)** |
|---|---|---|---|---|---|---|---|---|---|
| **Bivalves** | | | | | | | | | |
| *Dreissena polymorpha* | No | No | No | Yes | Yes | No | Inconclusive | Limited | No |
| *Scrobicularia plana* | No | Inconclusive | No | Inconclusive | No | No | Yes | Yes | No |
| *Crassostrea gigas* | Yes | No | Yes | No | No | Yes | Yes | Yes | Yes |
| *Ostrea edulis* | Yes | No | Yes | No | No | Yes | Yes | Limited | No |
| *Mytilus edulis* | No | Inconclusive | No | Inconclusive | Yes | No | Inconclusive | Yes | No |
| **Gastropods (marine/estuarine)** | | | | | | | | | |
| *Thais clavigera* | No | Yes | No | Yes | No | No | Yes | Yes | Yes |
| *Babylonia japonica* | No | No | Yes | No | No | Yes | Yes | Yes | No |
| *Nucella lapillus* | No | No | No | No | No | Inconclusive | Yes | Yes | Yes |
| *Nassarius reticulatus* | No | No | No | No | No | No | Yes | Yes | Yes |
| *Ilyanassa obsoleta* | Inconclusive | No | No | No | No | No | Yes | Yes | Yes |
| *Littorina littorea* | No | No | No | No | No | No | Yes | Yes | Yes |
| *Hydrobia ulvae* | No | No | No | Yes | Yes | No | Yes | Yes | No |

| **Species** | **Easily bred in the lab** | **Cheap to conduct tests** | **FLC/MGS* available** | **Compact space require-ments** | **Rapid generation time** | **large number of offspring produced in the lab** | **Apical endpoints easy to assess** | **Known sensitivity to some EDCs**** | **Significant information on culture & lab testing available (in English)** |
|---|---|---|---|---|---|---|---|---|---|
| *Crepidula fornicata* | Yes | No | Yes | No | Yes | Yes | Yes | No information | No |
| *Haliotis* spp. | No | No | Yes | No | No | Yes | Yes | Yes | No |
| **Gastropods (freshwater)** | | | | | | | | | |
| *Theodoxus fluviatilis* | No | No | No | No | Yes | No | No | Limited | No |
| *Potamopyrgus antipodarum* | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | Yes |
| *Marisa cornuarietis* | Yes | No | Yes | No | Yes | Yes | Yes | Yes | Yes |
| *Bithynia tentaculata* | No | No | No | No | Yes | No | Yes | Limited | No |
| *Viviparus viviparus* | No | No | No | No | Yes | No | Yes | Limited | No |
| *Lymnaea stagnalis* | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| *Planorbarius corneus* | Yes | No | No information | No | Yes | Yes | Yes | Limited | No |

* FLC (full life-cycle study), MGS (multi-generation study)

** Note that in several cases, experience of sensitivity to EDCs is limited to triorganotins

### 1.21 Strengths and weaknesses of different test methods and endpoints

As in other groups of organisms, there are two fundamental types of long-term apical test which can be conducted with molluscs. These are partial life cycle reproduction and/or developmental tests, and full life cycle tests including two-generation tests (see Figure 7-1). Like in fish (OECD, 2008), it is possible to measure a range of apical endpoints in tests of this type. It is also possible to measure some biochemical (e.g. hormone titres) and histopathological (e.g. gonad development) endpoints in life cycle tests with molluscs. These so-called biomarkers are summarised in section 1.21.3 but will not be considered further except two (imposex/intersex and vitellin induction) because there is generally insufficient knowledge of modes of action of EDCs in molluscs, or of molluscan endocrinology, to permit reliable interpretation.

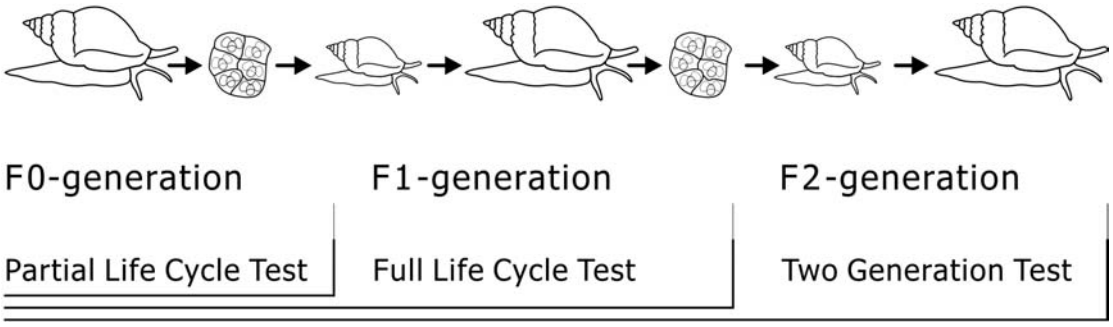


Figure 7-1: Flow chart of molluscan life cycle tests illustrating the main types.

#### 1.21.1 Partial life cycle tests

The partial life cycle test type about which perhaps most is known involves exposing adults to the test substance (generally dissolved in water, but with some species, e.g. *P. antipodarum*, it is also possible to expose them via sediments), and then measuring *inter alia* reproductive output in terms of numbers of eggs or developing embryos. In some cases (e.g. *M. cornuarietis* exposed to TPT – Schulte-Oehlmann *et al*., 2000; *L. stagnalis* exposed to fomesafen – Jumel *et al*., 2002; *L. stagnalis* exposed to vinclozolin - Lagadic, 2009), EDC exposure causes a reduction in fecundity, while in others (e.g. gastropods such as *P. antipodarum* exposed to EE2 – Jobling *et al*., 2004; and *M. cornuarietis* exposed to OP – Oehlmann *et al*., 2000) there have been observations of increases in fecundity when exposed to estrogens or their mimics (the so-called super-feminisation effect). Both effects can be considered as potentially adverse. In particular, although increased fecundity might be regarded as beneficial, it appears to be accompanied (at least in *P. corneus* – Clarke *et al.*, 2009; Ed Routledge pers. comm.) by smaller egg size and consequently reduced fitness of the F1 offspring. A negative relationship between clutch size and individual size at hatching is observed in several gastropods, including *L. stagnalis*. Moreover, a positive correlation was observed in this species, between adult size and fecundity (Coutellec and Lagadic, 2006). Therefore, if an EDC reduces clutch size, this may lead to an advantage for the hatchlings, in terms of size, and if this advantage is maintained through individual growth, it may thus result in higher fecundity when maturity is reached. In hermaphrodites, an impairment of the male function may lead to increased fecundity, due to a trade off in sex allocation. This may also occur due to an impairment of individual growth. In *Lymnaea stagnalis*, Koene *et al*. (2006) showed such negative relationships between male and female functions and between female function and growth, in relation to mate availability (which may partly involve allohormone transfer). If an EDC affects growth or the male function, it may thus indirectly increase fecundity.

With some species (e.g. bivalves such as *C. gigas* and gastropods such as *L. stagnalis*), it is also possible to easily measure interference with embryonic development by recording the proportion of normal larvae in comparison with those that are morphologically deformed (e.g. Nice *et al*., 2000; Lalah *et al*., 2007).

The other main type of partial life cycle test with molluscs covers the period of sexual development. This begins with fertilised embryos or juveniles, and can continue to sexual maturity, measuring a range of developmental endpoints including mortality, hatching success, growth rate, sex ratio (in appropriate species) and presence/absence of intersex individuals. There are fewer examples of this approach, but Tillmann *et al*. (2001) exposed juvenile (sexually immature) *M. cornuarietis* to the anti-androgen vinclozolin for 3 months and showed that the lower concentrations of the treatment (unbounded LOEC = 30 ng/l) temporarily delayed the onset of sexual maturity in males. In work with the bivalve *C. gigas* (described in more detail below for full life cycle procedures because this study went on to examine the survival of the F1 generation), Nice *et al*. (2003) showed that exposure of larvae to low concentrations of NP biased the sex ratio of the resulting adults towards female, and increased the incidence of hermaphrodites. Unfortunately, there are insufficient data to indicate whether this type of partial life cycle test starting with larval or juvenile molluscs is consistently more or less sensitive to EDCs than the type which begins with adults. However, PLC experiments in which *P. antipodarum* was exposed to the polycyclic musk HHCB have shown that juvenile growth, time to first reproduction, and reproduction itself are more sensitive endpoints than adult survival and growth (Pedersen *et al*., 2009).

### 1.21.2 Full life cycle tests

Full life cycle tests essentially combine the two types of partial life cycle test, and run from F0 adults through to F1 adults (or to F2 adults in the case of 2-generation tests), or from F0 fertilised eggs to F1 eggs or embryos. As with fish tests, there is a presumption that full life cycle tests with molluscs will cover most or all modes of EDC action, but there is insufficient experience with this approach in molluscs to judge whether it is indeed more sensitive to EDCs than either of the partial life cycle procedures. Czech *et al*. (2001) and Leung *et al*. (2007) have both used *L. stagnalis* in full life cycle experiments. Czech *et al*. (2001) started with adults and studied growth, mortality, fecundity, hatching rate and histopathology. They then allowed groups of F1 offspring to grow and measured the same endpoints in that generation (after a maximum of 7 months). The most sensitive NOECs were 24 ng/l for TBT, 10 ng/l for MT and β-sitosterol, and 10 µg/l for NP, which does not suggest greater sensitivity than shorter-term tests with molluscs. For example, the lowest reported LOEC for imposex induction in relatively short-term experiments with *Nucella lapillus* is 2.5 ng/l (ACP, 1994). In contrast, Leung *et al*. (2007) started their test of TBT with *L. stagnalis* egg ropes, measuring hatching success, growth, developmental abnormalities, mortality and finally fecundity of the adults (after about 6 months). Their NOEC for developmental effects was 10 ng TBT/l (i.e. similar to Czech *et al*., 2001), but their calculated NOEC for reduced population growth was much higher (2.7 µg/l), suggesting that *L. stagnalis* is not very sensitive to TBT compared with some other species (e.g. *Physa fontinalis*, where the population NOEC is only 100 ng TBT/l – Leung *et al*., 2004). Oehlmann et al. (2000) analysed the sensitivity of *M. cornuarietis* for BPA and OP in partial life cycle tests with adults (for 5 months) and in a 12 months full life cycle test, starting with an exposure of freshly produced eggs. They found no evidence for a higher sensitivity in the full life cycle test for both compounds. The response at the lower test concentration (1 µg/l) was almost identical with a slightly higher incidence of females with oviduct malformations in the full life cycle test while at the higher test concentration (100 µg/l) the enhancement of clutch and egg production was less striking in full life cycle tests.

Further life cycle experiments with *M. cornuarietis* were conducted by Tillmann *et al*. (2001), where adults were exposed to EE2 or MT and allowed to breed, and the offspring then reared to adulthood over 12 months in a variety of treatments which included the anti-androgen CPA. Both EE2 and MT (at the very high concentration of 500 ng/l) were able to induce imposex in the female F1 offspring, and it was shown

that CPA could successfully antagonise the effects of EE2 but not of MT. Penis sheath length was also measured, but not the more usual life cycle endpoints such as hatching success or fecundity. It is difficult to directly compare this test approach with that of Czech *et al*. (2001) due to the different endpoints measured, but it should be noted that the life cycle test with *M. cornuarietis* took 12 months, whereas that with *L. stagnalis* only took about 6 months. For *P. antipodarum*, there is limited experience showing that it is possible to conduct PLC tests with adult and juvenile phases exposed in parallel (Jensen and Forbes, 2001; Pedersen *et al*., 2009). This could shorten the time needed to detect effects on the full life cycle by approximately 4 weeks if the test is started with newly hatched juveniles, but of course might miss effects triggered in juveniles but not expressed until adulthood or in the F1 generation.

Finally, Nice *et al*. (2003) conducted life cycle experiments with the bivalve *C. gigas*, starting with developing larvae (exposed to NP for just 48 h), whose growth was regularly measured, and which were allowed to grow to adulthood and their sex ratio determined. Various crosses between exposed and control adult animals were then conducted by *in vitro* fertilisation, and survival of the resulting larvae was recorded. This procedure took 10 months. The lowest concentration tested (1 µg NP/l) had no effects on growth, but shifted the sex ratio of adult animals towards female and increased the number of hermaphrodites. Furthermore, this treatment also severely reduced survival (at 48 h post-fertilisation) of all crosses involving gametes (either eggs or sperm) derived from parents that were exposed as larvae. These data suggest that this type of life cycle experiment with *C. gigas* is considerably more sensitive to NP than the life cycle experiments conducted by Czech *et al*. (2001) with *L. stagnalis*, but they also took longer to conduct.

### 1.21.3 Biomarkers

As well as measuring apical endpoints such as fecundity, fertilisation success, growth and embryonic development in toxicological experiments, there are, as indicated above, several possible biomarkers which have been used to characterise exposure to and effect of EDCs in adults (and in juveniles if they can provide sufficient biomass for analysis). Although most of these are not recommended for use at this time, due to uncertainty about modes of action, two have been widely employed and should be considered.

The first of these is imposex in gastropods, and intersex as the related condition in littorinids, both of which were first reported as responses to triorganotins (Bryan *et al*., 1986; Bauer *et al*., 1995; Matthiessen *et al*., 1995), although imposex can also be induced by exposure to androgens such as T and MT, to aromatase inhibitors such as letrozole, formestane or flavone, and to comparatively high concentrations of estrogens such as EE2 (Bettin *et al*., 1996; Tillman *et al*., 2001; Duft *et al.*, 2007; Oehlmann *et al.*, 2007). In imposex, as has been observed for more than 150 prosobranch species (Matthiessen *et al*., 1999), the female genital system is superimposed by male sex organs (penis and vas deferens), while intersex in *Littorina littorea* is characterised by masculinisation of the female pallial organs and ultimately by their transformation into a prostate gland. Both of these processes result in sterility if sufficiently far advanced, so it can be reasonably argued that they are directly relevant for risk assessment, and hence of potential value in apical testing. However, it should be noted that the only one of the species with current potential for test standardisation which is known to produce the imposex response is *M. cornuarietis* (Schulte-Oehlmann *et al*., 1995, 2000; Tillmann *et al*., 2001). Another disadvantage of measuring imposex is that it requires a skilled microscopic examination of morphological changes.

As described above, abnormal numbers of intersex or hermaphrodite individuals can also be produced in *C. gigas*, although this has been reported as a consequence of exposure to NP, not organotins (Nice *et al*., 2003). In this case, sex can be determined by simple microscopic examination of gonad biopsies, and hermaphrodites with both types of gamete (a number of which are found in normal adults) can be easily recorded at the same time.

Another biomarker which has been used fairly widely in exposures of molluscs to estrogens and their

mimics is induction of vitellin as a yolk precursor and thus an analogue to vitellogenin in fish. In vertebrates, vitellogenin induction is known to be under the direct control of estradiol, and this process can be taken over by estrogen-mimics. In molluscs, vitellin can also be induced by estrogenic hormones (e.g. *Patinopecten yessoensis* exposed to E2 – Osada *et al*., 2003) and their analogues or mimics (e.g. *Lymnaea stagnalis* exposed to EE2 - Segner *et al*., 2003; *Mya arenaria* injected with E2, NP or PCP – Blaise *et al*., 1999; *Mytilus edulis* exposed to alkylphenols – Ortiz-Zarragoitea and Cajaraville, 2006), but the precise mode of action is unknown, and it is therefore premature at present to assume that its induction is diagnostic solely of exposure to estrogens or other EDCs. The relationship of vitellin induction to apical endpoints in molluscs such as reproductive success is also unclear so its use in mollusc life cycle experiments is not recommended at this time as an exclusive parameter.

### 1.21.4 Conclusions about test methods and endpoints

The most widely used mollusc long-term toxicity technique is to expose adults to a test substance and measure reproductive endpoints such as fecundity in a partial life cycle (PLC) test, possibly amended by further parameters like hatching success or larval survival. There is less experience with the alternative PLC approach of starting with eggs or larvae and measuring sexual differentiation or other developmental endpoints. The available data are insufficient to give a clear steer as to which PLC approach is the more sensitive to EDCs. Furthermore, the experience with mollusc full life cycle (FLC) tests is too limited to support or refute the contention that they are more sensitive to EDCs than PLC tests, although the more extensive data on fish suggests that FLC tests tend to be more sensitive than PLC tests to EDCs (Crane and Matthiessen, 2007). Some unpublished data are available for a comparison of effects observed in *L. stagnalis* exposed to the herbicide diquat in PLC and FLC tests (L. Lagadic pers. comm. 2009), although it should be noted that this substance probably does not act as an EDC in molluscs. Finally, at the current state of knowledge, the use of biomarkers in tests of either type cannot be recommended for standardisation as sole endpoints due to the uncertainty about molluscan endocrinology and the modes of action of EDCs in this group of organisms. However, there may be scope in future for the development of gene expression biomarkers in species such as *L. stagnalis*, for which sequences of several candidate genes are available (e.g. Lagadic *et al*. 2007, and GenBank database).

Given these experiences and uncertainties, and bearing in mind the recommendations made in section 1.20 concerning suitable test species, there is a case for optimising and validating partial life cycle test protocols with one or more of the gastropods *P. antipodarum* and *L. stagnalis*, which start exposure at the adult stage, and measure apical reproductive endpoints such as fecundity and fertility. Further research is necessary to decide the optimum mix of endpoints in such PLC tests which will provide maximum sensitivity to a range of ‘weak’ and ‘strong’ EDCs, and to define the optimum exposure time, although draft procedures are described below.

The big unknown is whether mollusc FLC tests are consistently more sensitive to EDCs than PLC tests, and therefore worth optimising and validating, although there are theoretical reasons for expecting the FLC approach to be more sensitive. Given the huge range of chemical properties and modes of action which partly determine the most sensitive lifestage, it seems likely that both PLC and FLC tests will be needed in particular circumstances. Both *L. stagnalis* and *C. gigas* show promise for use in FLC testing, and draft protocols incorporating many of the apical endpoints discussed above are described in the next sections, but more research using the same group of ‘weak’ and ‘strong’ EDCs is clearly necessary to establish beyond doubt whether either of these protocols is indeed more sensitive than the *P. antipodarum* or *L. stagnalis* PLC tests. To this end, it would be particularly useful if within-species comparisons could be made between the *P. antipodarum* and *L. stagnalis* PLC and FLC tests, although this would require suitable *P. antipodarum* FLC and *L. stagnalis* PLC protocols to be established. Indeed, it could be argued that within-species comparisons of this type are the only fair test of whether FLC procedures are consistently more sensitive than PLC methods.

## 1.22 Candidate partial life cycle test methods

### 1.22.1 The test organism *Potamopyrgus antipodarum*

*Potamopyrgus antipodarum* (GRAY 1843) belongs to the clade Mollusca, class Gastropoda, sub-class Streptoneura, order Mesogastropoda, family Hydrobiidae. The snail originates from New Zealand, but has been introduced to many parts of the world including Europe and North America. It inhabits running waters from small creeks to streams, lakes and estuaries, where it often reproduces in large quantities (Cope and Winterbourn, 2004; Fretter and Graham, 1962; Kinzelbach, 1995; Roth, 1987). The average shell height of adult snails varies according to the environmental conditions but generally is in the range of 3.5-5.0 mm (Figure 7-2).

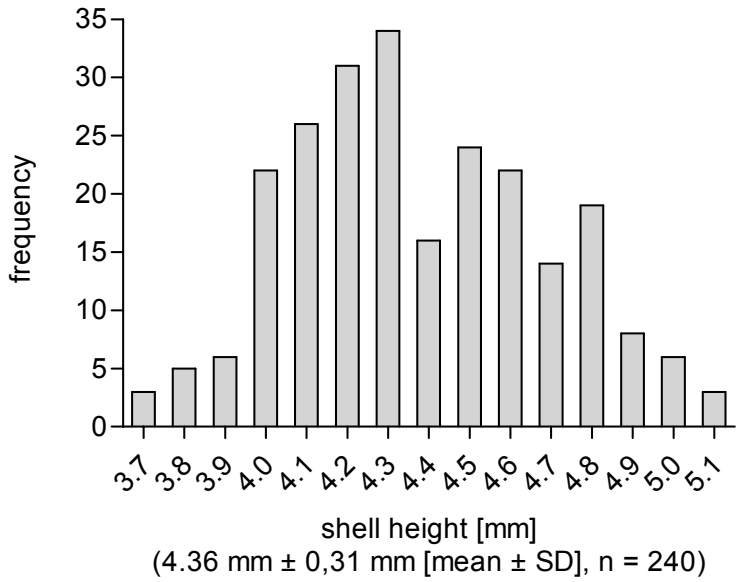


Figure 7-2: *Potamopyrgus antipodarum*. Shell height distribution in a sample of 240 control specimens from laboratory culture and experiments (Oehlmann, unpublished data).

Though predominantly living in freshwater, *P. antipodarum* is an euryhaline species able to survive and reproduce in brackish water with a salinity up to 15‰ (Jacobson and Forbes, 1997). Mudsnails prefer living in or on soft sediments in standing or slow running waters as well as in estuarine slack water areas. The species feeds on detritus, algae and bacteria, which are rasped from the surface of plants, stones or the sediment. In their area of origin, biparental populations sympatrically coexist next to monoparental populations, reproducing parthenogenetically. European populations consist almost entirely of females (Ponder, 1988; Wallace, 1979, Gérard et al., 2003). In this way a single snail is capable of establishing an entire population.

DNA fingerprinting studies on British and Danish populations have supported the notion of three clonal lineages, named A, B, and C, with A dominating freshwater habitats in Europe, whereas B and C were exclusively found in coastal, brackish-water habitats (Hauser et al., 1992; Jacobsen et al., 1996; Städler et al., 2005). Recent surveys of British populations using highly polymorphic microsatellite loci are concordant with these earlier molecular studies and have suggested the existence of a rare fourth strain D

(Weetman et al., 2002). The three main European clonal lineages A-C exhibit ecophysiological differences, and strains A and B appear to have 'generalist' qualities (Hughes, 1996; Jacobsen and Forbes, 1997). Additionally, the clones exhibit differences in life history traits and sensitivity to cadmium (Jacobsen and Forbes, 1997; Jensen and Forbes, 2001; Jensen et al., 2001). Differences in sensitivity for organic compounds among clones have not been investigated so far.

**Principle of the test**

The chosen protocol to be discussed in detail refers to a 56-day exposure test via water and/or sediment of sexually mature *Potamopyrgus antipodarum*. The protocol has at least partially been applied to exposures with native sediments (environmental mixtures), pharmaceuticals (EE2, tamoxifen, CPA, letrozole), industrial chemicals (BPA, NP, OP, TBT, TPT), personal care products (UV svreens 3-benzylidene-camphor and 3-(4'-methylbenzylidene)-camphor) and a range of heavy metals (including Cd and Cr) (Duft et al., 2003a & b; Jobling et al., 2004; Mazurová et al., 2008; Oetken, 1999; Schmitt et al., 2008; Schulte-Oehlmann, 1997) and allows for the assessment of chronic effects on reproduction (fecundity) and survival (mortality). The test focuses on reproduction as an integrative endpoint. Changes in the number of offspring compared to a control can be induced by several substances known to have an impact on reproduction. The test addresses the assessment of hormonally active substances but is not exclusively sensitive to EDCs and is equally suitable for the detection of adverse effects on reproduction mediated via other modes of action.

A brief overview of the candidate protocol is presented below:

Adult *Potamopyrgus antipodarum* (exclusively parthenogenetically reproducing females) are exposed to a concentration range of the test substance. The ambient water or sediment is dosed with the test substance. Adult snails are subsequently introduced into the test beakers (in the case of a sediment test, sediment and water concentrations have to be equilibrated beforehand). Survival of the snails is determined at the end of the test, while reproduction is examined in 40 exposed snails per treatment group (4 replicates with 10 snails each) after 28 and 56 days of exposure, respectively. Snails are narcotized in $MgCl_2$ (2.5% in distilled water) for 45 to 90 min prior to analysis. Shell and aperture height are measured before cracking the shell by a small vice or combination pliers and shell fragments are removed. Thereafter, embryos can be seen through the translucent epithelium of the brood pouch. The latter is opened carefully, embryos are removed and their number counted using a dissecting microscope. As the embryos are very small and information on embryo normogenesis in this species is rather fragmentary, developmental variables will not be considered in detail, but developmentally more advanced embryos (with shells) are distinguished from less advanced embryos (without shells) and counted separately. Counting of embryos allows for an assessment of individual fertility in terms of ability to reproduce in this species. The evaluation of fecundity (quantification of actual, visible offspring) is also possible, but would require the elaboration of a completely different and more complex test protocol. In the study of Pedersen et al. (2009) the released progeny were counted. This could indeed be a possibility for a less destructive test design but only for the testing of sediments. When testing via water in a semi-static exposure design, the whole medium has to be changed, which might be a stress factor and lead to the holdback of progeny in the brood pouch of *P. antipodarum*. In a flow through system the released progeny can hardly be quantified as a loss of released offspring due to the constant water flow cannot be ruled out and is likely to happen. Moreover, and in contrast to egg-laying gastropods, the choice of the ovoviviparous *Potamopyrgus* species will not provide insight into hatching success as complete embryonic development takes place inside the maternal organism.

**Material and Methods**

Equipment

Test vessels and other apparatus used should ideally be made of glass or other materials chemically inert to the test chemical. All glassware must be washed thoroughly to remove any contaminants. Before washing, glassware should be sterilised by autoclaving. An overnight soak of all test equipment in DECON$^{TM}$ 90 (5% DECON 90 in tap water) or similar decontaminant is required, the final rinse being in deionised distilled water.

The following equipment is required:

- 1000 mL glass beakers
- glass pipettes
- oxygen meter
- pH meter
- conductivity meter
- thermometer
- stereomicroscope
- climate chambers or temperature regulated room with temperature indicator
- dissecting dish and dissecting instruments

Test medium: water

Reconstituted (synthetic) water should be used as test medium and be composed of 2.5 g sea salt (e.g. Tropic Marin®) and 1.5 g sodium hydrogen carbonate ($NaHCO_3$), dissolved in 10 litres deionised water. The reconstituted water is prepared and stored in a 50 litre glass aquarium. Before use it has to be aerated for at least 24 hours and circulated by a water pump continuously to guarantee complete dissolution of all ingredients. Special care must be taken not to take water from water pipes made of copper, since the corresponding Cu concentrations may invoke acute snail mortality.

Test medium: sediment

Since uncontaminated natural sediments may not be available at requested quantities, and indigenous organisms as well as micropollutants might impact test results, it is recommended to use an artificial sediment. For the assessment of native sediments it is recommended to deep-freeze them before testing to minimize infauna effects. Concentrations of ammonia, nitrite and nitrate in the test beakers should be regularly monitored as especially ammonia and nitrite may impact reproduction in *Potamopyrgus* at concentrations above 2 mg/L and 5 mg/L, respectively.

A suitable artificial sediment is composed of:

- 97.5% (dry weight) quartz sand (fine sand, mean grain size 50-200 µm)
- 2.5% (dry weight) powdered leaves of beech (*Fagus sylvatica*) or alternatively alder (*Alnus glutinosa*) (use only brown-coloured fallen leaves)

Test conditions

Water parameters should be as follows:

- temperature 16 ± 1°C
- pH: 8.0 ± 0.5
- oxygen concentration: > 6 mg/L
- oxygen saturation: > 60%
- conductivity: 770 ± 100 µS/cm

Test vessels should contain 800 ml of reconstituted water. Water should be aerated through glass pipettes (Pasteur pipettes). Dissolved oxygen content should be kept above 60%, however the test vessels should be aerated as little as possible to avoid stripping of test chemicals. The water in each test vessel should be completely renewed at least three times per week (preferably synchronized with feeding interval). Water quality parameters (pH, dissolved oxygen content, conductivity, temperature and total nitrite content) should be measured before the water renewal in all test vessels. Additional measurements of ammonium and nitrate should be made if necessary. Constant photoperiods of 16 hours light to 8 hours darkness should be maintained at an intensity that does not exceed 500 lux (lumens/m2) at the water surface.

Feeding of test animals

Feeding should preferably be done daily, but at least three times per week (at two day intervals) with finely ground commercial feed such as TetraPhyll® ad libitum (approx. 0.25 mg per animal).

Test concentrations and replicates

Chemical stability analyses should be performed in the run-up to the test to check for stability of the test substance, as degradability may have implications for choice of test media and substance renewal intervals. Usually the test substance will be added with every renewal of the exposure water except in the case of sediment tests, when renewal of spiked sediment is not usually necessary.

At least 5 concentrations and a control group with at minimum four replicates per concentration should be tested in a geometric series with a factor between concentrations not exceeding 2.2. In the run-up to the definitive test the performance of range finding studies will help to select appropriate test concentrations.

If a solubilizer has been applied, a solvent control using the same amount of solvent as in the treatments should be used. The amount of solvent in test concentrations should be as small as possible, not exceeding a maximum concentration of 0.01% (100 µl/l or 100 mg/l). Dimethyl sulfoxide (DMSO) is recommended but the selection of the appropriate solvent depends on the chemical constitution of the test substance. If ethanol or other readily biodegradable solvent are used, the maximum concentration should be 0.003% to avoid bacterial and fungal growth and biofilm development in the test vessels. Further information on appropriate organic solvents may be obtained from Jarosz and Weltje (2008).

In case of a sediment test (Duft et al., 2003a & b, 2007), each test solution is mixed with quartz sand. If needed, the test substance should be dissolved in a suitable organic solvent (e.g. ethanol, ethylacetate). In order to soak the quartz sand completely, a solution volume of 0.24 ml/g sand has proven to be sufficient. It should be ensured that the test chemical added to the sediment is equally distributed within the sediment. After spiking of the sediment, the organic solvent has to be evaporated completely before the sediment is covered with a layer of water. It is desirable to allow sediment-water partitioning of the test substance (ASTM, 2000; SETAC, 1993; US-EPA, 2000). Equilibration time should be 2 to 7 days and be carried out under the same temperature and aeration conditions as used in the test.

**Biological methods**

Test organisms should be taken from a healthy breeding stock (i.e. without any sign of stress and parasitism which is normally revealed by enhanced mortality and comparably poor fecundity checked

against the maxima and minima of the annual reproductive cycle). Test organisms must be maintained in culture conditions (light, temperature, medium and feeding) similar to those of the laboratory breeding stock. Shell heights of laboratory populations may vary considerably. Therefore, sexual maturity of the test organisms, which is normally obtained at a shell height around 3.5 mm (Duft, 2004), should have been reached. Duft et al. (2007) recommend the use of snails with a shell height between 3.7 and 4.3 mm, aged about 3–6 months. Sexually immature snails that may eventually occur in a sample during a test have to have to be excluded from analysis.

25 adult snails have to be allocated randomly using forceps to each replicate test vessel containing the exposure water/sediment. The test vessels should be checked for abnormal test conditions (e.g. bacterial or fungal growth) or behaviour of test organisms (for example avoidance of water) at least three times per week. Reproductive success (fecundity) of 10 snails per replicate should be analysed following on day 28 and of all surviving snails on day 56 of exposure to the tested substance.

Test organisms should be narcotised for 45-90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$) dissolved in deionised or distilled water prior to dissection.

Shell and aperture height of snails should be measured via stereomicroscopical analyses with an ocular micrometer. Thereafter shells should be cracked with a small vice or combination pliers. Soft bodies are dissected by removing the shell with pointed forceps in a solution of magnesium chloride hexahydrate (2.5%). The brood pouch of the snails should be opened carefully with a dissecting needle, embryos removed and counted, distinguishing shelled and unshelled embryos.

**Data and reporting**

Validity criteria

Oxygen concentration in the water should not be below 60% of air saturation value at test temperature before water renewal.

The pH of the water should be in the basic measuring range. Because of shell decalcification the pH must not fall below pH 6.5.

The concentration of the test substance should be measured to ensure that the substance concentration has been maintained over the test period.

Mortality in adult control snails should not exceed 20%.

Appropriate positive controls (e.g. 25 ng EE2/l and 250 ng TBT as Sn/l) have to be applied to demonstrate the responsiveness of the snails to EDCs (comp. vom Saal and Welshons, 2006).

Treatment of results

When planning the test, it should be taken into consideration whether the aim is to calculate a NOEC/LOEC (by use of ANOVA or comparable nonparametric tests) or ECx values (by use of (non-)linear regression). The demand for replicates is higher if the ANOVA statistic is used whereas the (non-)linear regression generally demands more concentrations.

Endpoints to be evaluated statistically are mortality, the total number of embryos and the number of unshelled embryos per female. Data are recorded e.g. in a spreadsheet. The mean, and variability parameters such as standard deviation or standard error of the mean, are calculated for the shell height, aperture height, number of embryos with shell, number of embryos without shell, and the total number of embryos.

ECx (effect concentration)

ECx values are calculated using an appropriate statistical model (e.g. logistic or Weibull function). An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation. To compute the

EC50 or any other ECx, the individual replicates should be used as input for the regression analysis.

NOEC/LOEC (No Observed Effect Concentration/Lowest Observed Effect Concentration)

If a statistical analysis is intended to determine a NOEC/LOEC value, a one-way analysis of variance (ANOVA) and a multiple comparison procedure (e.g. Dunnett's or Williams' test) is recommended for embryo numbers. The latter allows definition of the treatment concentration that characterizes significant differences for reproduction parameters compared to the control. One-way analysis of variance is based on the assumptions that the observations are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using the ANOVA. If data sets do not comply with these requirements the normality and the homogeneity of variances can be increased by the following transformation of data before testing (cf. section 6.5.4 for details):

- quantal responses expressed as a percentage (e.g. survival or mortality, proportion of females or males) should be arcsine-square root transformed,
- counts (e.g. numbers of produced eggs and clutches or number of embryos in the brood pouch) should be square root transformed,
- continuous data (e.g. growth or weight measures) should be transformed to the natural logarithm.

Following transformation, data sets have to be tested again for normal distribution and homogeneity of variances. Gaussian distribution can be verified e.g. by the Kolmogorov-Smirnow normality test, whereas Bartletts's test represents a method for checking homogeneity of variance. In case data do not meet the assumptions for ANOVA, non-parametric procedures such as the Kruskal-Wallis-Test should be used. For the analysis of mortality (quantal data) the Fisher's exact test is recommended if the mortality of the individuals is independent. If individuals are living in the same test vessel, this assumption is violated. If data follow a monotone concentration response, a step-down trend test such as Cochran-Armitage and Jonkheer should be used to analyse quantal responses.

**Reporting requirements**

Laboratory reports should include the following information:

Testing site

- Identification of the laboratory, dates of testing and key personnel involved

Test substance

- chemical identification of the substance (name, structural formula, CAS number, etc.) and organic solvent (if any) including purity, manufacturer, lot or batch number
- relevant physicochemical properties (e.g. water solubility, vapour pressure)
- analytical method for quantification of the test substance where appropriate
- appropriate negative and positive controls

Test species

- scientific name, source and culture conditions

Test conditions

- test procedure applied including photoperiod and light intensity, test design (e.g. test concentration used, number of replicates, number of snails per replicate)
- method of test substance pre-treatment, stock solution preparation and substance delivery system
- nominal test concentrations, details of sampling for chemical analysis of the test concentrations

and the analytical methods by which the concentrations were analysed (including recovery and detection limit)
- information on the test conditions regarding pH, salinity, hardness, conductivity, temperature and dissolved oxygen concentration and saturation, nitrate, nitrite and ammonium concentrations
- information on feeding (e.g. source and type of food, quantity, feeding interval).
- compliance with validity criteria

Results

- references to chemical and statistical procedures (including assessment of the assumptions on which they are based)
- tabular and graphical (when appropriate) presentation of all measured and calculated endpoints
- tabular presentation of measured test concentrations including quality control samples
- NOEC/LOEC (including mean values, standard errors, error degrees of freedom and the least significant difference, or standard error of the difference) or ECx values (including the mathematical equation of the model used to estimate ECx values, the slope of the concentration response, confidence limits around the slope and the ECx values, assessment of goodness-of-fit and if possible assessment of appropriateness of the model applied) should be reported and p-values should be specified
- any deviation from the protocol should be accompanied by a discussion on possible impact on the study results

### 1.23 Candidate full life cycle test methods

A full life-cycle test could be developed for use with the great pond snail *Lymnaea stagnalis* (a) or the Pacific oyster *Crassostrea gigas* (b). Given some modification of methods, *Potamopyrgus antipodarum* may also be considered for the performance of a full life-cycle test.

#### 1.23.1 The test organism *Lymnaea stagnalis*

*Lymnaea stagnalis* (Linnaeus 1758) belongs to the clade Mollusca, class Gastropoda, sub-class Euthyneura, order Basommatophora, family Lymnaeidae. The species is common in Europe and can be found throughout northern Asia and North America (Pfleger and Chatfield, 1983). It inhabits stagnant or slow-running waters and feeds on organic constituents of plant and animal origin and is known to be cannibalistic on occasion.

The average shell height of adult snails varies according to the environmental conditions (e.g. the volume of surrounding water) but generally is in the range of 20-60 mm. *L. stagnalis* (the great pond snail) is a simultaneous hermaphrodite but the male reproductive organs mature prior to the female ones (protandric hermaphrodite). During mating, an individual can only take the male or the female part. *L. stagnalis*, as all Basommatophora, can self-fertilize and appears to show no or very weak inbreeding depression (Puurtinen et al., 2001; Coutellec and Lagadic, 2006), while for other species such as *Physa acuta* (Jarne et al., 2000) and *Lymnaea peregra* (Coutellec-Vreto et al., 1998) a strongly reduced fitness under selfing has been shown.

Egg-masses are deposited on aquatic plants or in laboratory breeding stocks affixed to the glass surface of the aquaria. The number of eggs per clutch varies from 50 to 120 (Nichols et al., 1971). Renewal of the surrounding water is known to be a trigger for egg laying (Ter Maat et al., 1983).

**Principle of the test**

The protocol to be discussed in detail refers to a 31 week (217 day) full life-cycle exposure via water of sexually mature *Lymnaea stagnalis* (parental generation F0) and their offspring (first filial generation F1).

In principle, however, the test could be terminated at the point when the F1 snails reach adulthood (23 weeks), rather than allowing it to continue to the point (31 weeks) where F1 fecundity can be assessed. The protocol has been composed by merging the methods of several differently designed *Lymnaea* eco(toxico)logical studies (e.g. Bandow, 2009; Bandow and Weltje, 2008; Czech et al., 2001; Coutellec and Lagadic, 2006; De Schamphelaere et al., 2008; Leung et al., 2007; Pésing, 1993; Ter Maat et al., 2007; Weltje et al., 2003). Substances investigated in the above-mentioned studies included pesticides (the fungicide vinclozolin; the herbicide fomesafen; and the insecticide deltamethrin), pharmaceuticals (EE2, MT, tamoxifen, CPA), phytoestrogens (ß-sitosterol), industrial chemicals (BPA, TBT, NP, NP ethylene oxide) and heavy metals (Cu). The test protocol presented here allows for the assessment of chronic effects on reproduction (fecundity and fertility), development (hatching success) growth and survival (mortality). The test focuses on reproduction as an integrative endpoint. Changes in the quantity of eggs or compared to a control can be induced by several substances known to have an impact on reproduction. The test covers the assessment of hormonally active substances but is not exclusively sensitive to EDCs and is equally suitable for the detection of adverse effects on reproduction mediated via different modes of action.

A brief overview of the candidate protocol is presented below:

Adult *Lymnaea stagnalis* (F0) are exposed to a concentration range of the test substance. The ambient water is dosed with the test substance. Adult snails are subsequently placed in glass aquaria. Survival of the parental generation is determined at the end of the adult exposure of 8 weeks. Fecundity of the F0 generation (number of eggs and clutches produced per day, number of eggs per clutch) is examined daily for all sub-groups of 20 exposed adult snails. Egg clutches (F1 generation) are counted and individually transferred into 6-well-plates with the water being dosed with the same test substance and concentration as in the parental generation and hatching success is determined as an integrative parameter for fertilisation failure and embryonic mortality. Hatching normally takes place within 2-3 weeks after spawning but may be delayed until 6 weeks after spawning under chemical exposure. Additional impacts on embryo development can be measured by calculation of the mean hatching time ($HT_{50}$, hatching time for 50% of the individuals).

Once hatching is complete, 20 hatchlings obtained from clutches spawned at the end of the exposure period (8 weeks) are again transferred to 1000 mL glass beakers whose ambient water is dosed with the same test substance and concentration as the respective parental generation. Juveniles of the F1 generation are reared under the same test conditions until sexual maturity which is normally reached 3 months after hatching.

Whereas counting of egg clutches and number of eggs/clutch in the parental generation (F0) allows for the assessment of fecundity (in terms of visible quantity of the actual offspring) in *Lymnaea*, the evaluation of fertility (ability to reproduce, sexual maturity) is demonstrated in the F1 generation. Sexual maturity is expected to occur in controls at about a shell height of 21-24 mm (Zonneveld and Kooijman, 1989). Yet, size alone is not a reliable indicator of the maturity of snails exposed to chemicals. The variable “age at sexual maturity” thus cannot be reliably assessed in these tests, unless histologically verified (appearance of ripe gametes in the gonad). Nonetheless, the variable “age at first spawning” of the F1 generation can be recorded. Given the potentially inhibiting or stimulating effects of a test substance, time spans for sexual maturation can vary considerably. As soon as egg-laying is observed in one of the exposure groups, fecundity of the F1 generation (number of eggs and clutches produced, number of eggs per clutch) is determined daily for all sub-groups of 20 exposed F1 snails for a period of at least 8 weeks. Fecundity is assessed by counting of egg clutches and numbers of viable eggs per clutch. Egg clutches are removed from test chambers, counted, and transferred into new plates, with the water being dosed with the same test substance and concentration as in adults. Additional impacts on the duration of embryonic development in the F1 and F2 generation can be assessed providing the age of the clutches is known at the beginning of their exposure.

**Material and Methods**

Equipment

Test vessels and other apparatus used should ideally be made of glass or other materials chemically inert to the test chemical. All glassware must be washed thoroughly to remove any contaminants. Before washing, glassware should be sterilised by autoclaving. An overnight soak of all test equipment in a decontamination agent such as DECON$^{TM}$ 90 (5% DECON 90 in tap water) is necessary, the final rinse being in deionised distilled water.

The following equipment is required:

- 10 L glass aquaria for exposure of the F0 and F1 generation
- 6-well plates, 10 mL per well, for the exposure of egg clutches (one clutch per well)
- 1000 mL glass beakers (for exposure of young hatchlings)
- glass pipettes
- oxygen meter
- pH meter
- conductivity meter
- thermometer
- stereomicroscope
- climate chambers or temperature regulated room with temperature indicator

Test medium

Reconstituted (synthetic) water should be used as test medium and be composed of 2.5 g sea salt (e.g. Tropic Marin®) and 1.5 g sodium hydrogen carbonate ($NaHCO_3$), dissolved in 10 litres deionised water. The reconstituted water is prepared and stored in an aquarium with at least 200 L tank volume. Before use it has to be aerated for at least 24 hours and circulated by a water pump continuously to guarantee complete dissolution of all ingredients. Special care must be taken not to take water from water pipes made of copper, since the corresponding Cu concentrations may invoke acute snail mortality.

Test conditions

Water variables should be as follows:

- temperature 20 ± 1°C
- pH: 8.0 ± 0.5
- oxygen concentration: > 6 mg/L
- oxygen saturation: > 60%
- conductivity: 770 ± 100 µS/cm

Test aquaria for the F0 and F1 fecundity assay should contain 8 L of reconstituted water and should be covered with nets or glass plates to prevent snails from escaping. Water should be circulated by a water pump and aerated through glass pipettes (Pasteur pipettes). Dissolved oxygen content should be kept above 60%, however the test aquaria should be aerated as little as possible to avoid stripping of test chemicals. The water in each test aquarium should be completely renewed at least three times a week (preferably synchronized with feeding interval). Water quality variables (pH, dissolved oxygen content, conductivity, temperature and total nitrite content) should be measured (and if needed adjusted) before water renewal in all test aquaria. Additional measurements of ammonium and nitrate should be done if necessary. Constant photoperiods of 16 hours light to 8 hours darkness should be maintained.

Egg clutches sampled from the test aquaria are further exposed in 6-well plates with 10 mL exposure medium per well (one clutch per well). After hatching, young snails are transferred to 1000 mL test vessels with 800 mL of exposure medium. Water should be aerated through glass pipettes (Pasteur pipettes).

Feeding of test animals

Feeding should be done at least three times per week (at a two-days interval) with lettuce (organic quality washed in clean water) in combination of feeds such as TetraPhyll® and Tetramin® ad libitum. This implies the need to gradually increase the amount of delivered food during the test as F1 snails grow. Food should be provided only when the previously given amount has been consumed by the snails.

Test concentrations and replicates

Chemical stability analyses should be performed in the run-up to the test to check for stability of the test substance as degradability may affect renewal intervals of test media and substance. The test substance is added with every renewal of the exposure water.

At least 5 concentrations and a control group with at minimum five replicates (four snails per replicate) should be tested in a geometric series with a factor between concentrations not exceeding 2.2. In the run-up to the definitive test the performance of range finding studies will help to select appropriate test concentrations. If a solubilizer has been applied, a solvent control with the same amount of solvent as in the treatments should be used. The amount of solvent in test concentrations should be as small as possible, not exceeding a maximum concentration of 0.01% (100 µl/l or 100 mg/l). Dimethyl sulfoxide (DMSO) is recommended but the selection of the appropriate solvent depends on the chemical constitution of the test substance and sensitivity of test organisms and is usually determined in a precursor study. If ethanol or other readily biodegradable solvents are used, the maximum concentration should be 0.003% to avoid bacterial and fungal growth and biofilm development in the test vessels. Further information on appropriate organic solvents may also be taken from Jarosz and Weltje (2008).

**Biological methods**

Sexually mature test organisms with a shell height of 25-35 mm should be taken from a healthy breeding stock (i.e. without any sign of stress and parasitism which is normally revealed by enhanced mortality and poor fecundity). Test organisms must be maintained in culture conditions (light, temperature, medium and feeding) similar to those of the laboratory breeding stock. Shell heights of F0 animals could be recorded by stereomicroscopical analysis with an ocular micrometer or sliding calliper and must not show statistically significant differences between replicates. Therefore 20 adult snails of comparable size have to be allocated randomly to each exposure group (i.e. 4 snails per test aquarium if replicate number is 5). Adult snail loading should be at least 500 mL per individual.

The test aquaria should be monitored for abnormal test conditions (e.g. bacterial or fungal growth) or behaviour of test organisms (for example avoidance of water) at least three times per week. Dead snails should be withdrawn daily from the aquaria to prevent deleterious effects on survivors. Changes in snail density within a replicate have to be accounted for in the calculation of the amount of food provided per test vessel and in the calculation of fecundity. Reproductive success (eggs, clutches and eggs/clutch) should be analysed daily during at least a period of 8 weeks. Clutches are removed by razor blades from the glass surface, the egg number per clutch counted and transferred into 6-well-plates in which the water has been dosed with the same test substance and concentration as the respective parental generation. Hatching success (percent hatch per clutch) and time (mean hatching time, $HT_{50}$) of the F1 snails is determined for each replicate using the following procedure. The number of eggs in each clutch is determined (i) at the beginning of exposure to the contaminated water and (ii) after 35 days of exposure. Obtained values are used to calculate the percent hatch per clutch. If the $HT_{50}$ is to be calculated, then the number of remaining eggs in the clutch should be regularly monitored (e.g. every two days) from the beginning of clutch exposure until day 35.

20 F1 hatchlings per exposure group, obtained from clutches spawned at the end of the exposure period (8 weeks) are again transferred to 10 L glass aquaria (4 snails per aquarium if replicate number is 5) whose ambient water is dosed with the same test substance and concentration as the respective parental

generation. Juveniles of the F1 generation are reared until first spawning which normally takes place at an age of 3 months. The time to first egg-laying is recorded for all replicates. As soon as spawning occurs in one of the exposure groups, fecundity of the F1 generation (number of egg clutches produced, number of eggs per clutch) is determined as previously described for the F0 generation. Additionally, hatching success (percent hatch per clutch) and time (mean hatching time, $HT_{50}$) of the F2 snails is determined for all eggs and clutches produced during the first 4 weeks of the 8 week reproduction period of F1 snails within the following 2-3 weeks for each replicate and exposure interval.

**Data and reporting**

Validity criteria

Oxygen concentration in the water should not be below 60% of air saturation value at test temperature before water renewal.

The pH of the water should be in the basic measuring range. Because of shell decalcification the pH must not fall below pH 6.5.

The concentration of the test substance should be measured to ensure that the substance concentration has been maintained over the test period.

Mortality in the F0 and F1 adult control snails should not exceed 20%.

Reproductive rate of the controls for the F0 and F1 generation should be at least 0.3 egg masses/d per animal in the period between day 21 and 56

At least 70% hatching success in the control.

Appropriate positive controls have to be applied to demonstrate the responsiveness of the snails to EDCs.

Treatment of results

When planning the test, it should be taken into consideration whether the aim is to calculate a NOEC/LOEC (by use of ANOVA or comparable nonparametric tests) or ECx values (by use of (non-)linear regression). The demand for replicates is higher if the ANOVA statistic is used whereas the (non-)linear regression generally demands more concentrations.

Endpoints to be evaluated statistically are mortality, the cumulative number of egg clutches, average embryo number/clutch, time to first reproduction, development rate, mean hatching time. Data are recorded e.g. in a spreadsheet. The mean and variability parameters such as standard deviation or standard error of the mean, for the shell height, time to first reproduction, cumulative number of clutches, number of embryos per clutch, the total number of embryos and hatching time, are calculated.

ECx (effect concentration)

ECx values are calculated using an appropriate statistical model (e.g. logistic or Weibull function). An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation. To compute the $EC_{50}$ or any other ECx, the individual replicates should be used as input for the regression analysis.

NOEC/LOEC (No Observed Effect Concentration/Lowest Observed Effect Concentration)

If a statistical analysis is intended to determine a NOEC/LOEC value the one-way analysis of variance (ANOVA) and a multiple comparison procedure (e.g. Dunnett's or Williams' test) is recommended. The latter allows the definition of treatment concentration that characterize significant differences for reproduction/development parameters compared to the control. One-way analysis of variance is based on the assumptions that the observations are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using the ANOVA. If data sets do not comply with these requirements the normality

and the homogeneity of variances can be increased by the following transformation of data before testing (cf. section 6.5.4 for details):

- quantal responses expressed as a percentage (e.g. survival or mortality) should be arcsine-square root transformed,
- counts (e.g. numbers of produced eggs and clutches or number of embryos in the brood pouch) should be square root transformed,
- continuous data (e.g. growth or weight measures) should be transformed to the natural logarithm.

Following transformation, data sets have to be tested again for normal distribution and homogeneity of variances. Gaussian distribution can be verified e.g. by the Kolmogorov-Smirnow-T normality test, whereas Bartletts's test represents a method for checking homogeneity of variance. In case data do not meet the assumptions for ANOVA, non-parametric procedures such as the Kruskal-Wallis-Test should be used. For the analysis of mortality (quantal data) the Fisher's exact test is recommended if the mortality of the individuals is independent. If individuals are living in the same test vessel, this assumption is violated. If data follow a monotone concentration response, a step-down trend test such as Cochran-Armitage and Jonkheer should be used to analyse quantal responses.

**Reporting requirements**

Laboratory reports should include the following information:

Testing site

- Identification of the laboratory, dates of testing and key personnel involved

Test substance

- chemical identification of the substance (name, structural formula, CAS number, etc.) including purity, manufacturer, lot or batch number
- relevant physicochemical properties (e.g. water solubility, vapour pressure)
- analytical method for quantification of the test substance where appropriate
- appropriate negative and positive controls

Test species

- scientific name, source and culture conditions

Test conditions

- test procedure applied including photoperiod and light intensity, test design (e.g. test concentration used, number of replicates, number of snails per replicate)
- method of test substance pre-treatment, stock solution preparation and substance delivery system
- nominal test concentrations, details on sampling for chemical analysis of the test concentrations and the analytical methods by which the substance concentrations were analysed (including recovery and detection limit)
- information on the test conditions regarding pH, salinity, hardness, conductivity, temperature and dissolved oxygen concentration and saturation, nitrate, nitrite and ammonium concentrations
- information on feeding (e.g. source and type of food, quantity, feeding interval).
- compliance with validity criteria

Results

- references to chemical and statistical procedures (including assessment of the assumptions on which they are based)
- tabular and graphical (when appropriate) presentation of all measured and calculated endpoints
- tabular presentation of measured test concentrations including quality control samples
- NOEC/LOEC (including mean values, standard errors, error degrees of freedom and the least significant difference, or standard error of the difference) or ECx values (including the mathematical equation of the model used to estimate Ecx values, the slope of the concentration response, confidence limits around the slope, assessment of goodness-of-fit and if possible assessment of appropriateness of the model applied) should be reported and p-values should be specified
- any deviation from the protocol accompanied by a discussion on possible impact on the study results

### 1.23.2 The test organism *Crassostrea gigas*

*Crassostrea gigas* (Thunberg 1793) belongs to the clade Mollusca, class Bivalvia, order Ostreoida, family Ostreidae. The species is common in Europe and North America but originates from Japan and south-east Asia. In Europe the introduction of *C. gigas* was mainly driven economically as the species served for the replacement of the collapsed European oyster (*Ostrea edulis*) fishery (Nehring, 1999; Wolff and Reise, 2002).

Pacific oysters are meroplanktonic organisms: while the adults are benthic, the larvae are free-swimming. The species inhabits shallow water and intertidal environments of estuarine and coastal marine waters in depths between 2 and 8 metres, and tolerates mean salinity in the range of 23-35‰. Oysters are filter-feeders removing phytoplankton and suspended organic matter from seawater (Langdon and Newell, 1996).

*C. gigas* is a consecutive protandric hermaphrodite (first spawns as male). Depending on environmental conditions the species is able to change sex one or more times during its life span. It becomes sexually mature at approximately one year. Although adults are known to withstand temperature spans between -5ºC and 35ºC, spawning requires a temperature of 20 ± 2ºC. An increase in water temperature induces a few initial oysters to spawn, resulting in a series of spawning cascades in both sexes of neighbouring specimens. The release of gametes into the surrounding water is followed by external fertilization within the next 10-15 hours. Depending on water temperature, planktonic development of larvae takes 3 to 4 weeks until settlement. Oyster growth rate depends on food supply and temperature (Kennedy, 1996). During the first six months of life, oysters grow rapidly (up to 10 mm/month) but slowly throughout the rest of their life (Quast et al.. 1988). During 2-3 years of growth they can attain a maximum size of up to 8-9 cm (Gruner et al., 2000).

**Principle of the test**

The protocol to be discussed in detail refers to a 40 weeks (280 days) full life-cycle exposure via water of *Crassostrea gigas* larvae (filial generation F0) and their offspring (second filial generation F1). The protocol has been composed by merging the methods of several differently designed *Crassostrea* exposure studies (e.g. Geffard et al., 2002, 2003; His et al., 1997; Nice et al., 2000, 2003; Nice, 2005; Thain, 1992; Waldock and Thain, 1983). Concerning developmental variables, the protocol basically takes advantage of the oyster embryo bioassay developed by Woelke (1972) but also includes some details of the bivalve larval acute toxicity test as proposed under OPPTS 850.1055 (US-EPA, 1996). Regarding the assessment of reproductive parameters in adults, the protocol complies to a large extent with the methods described by Nice et al. (2003) and Nice (2005). Substances investigated in the above-mentioned studies mainly

included environmental mixtures from monitoring studies, industrial chemicals (NP, PAH mixtures, TBT) and heavy metals (Cd, Cu, Hg, Zn, Pb and their mixtures). The test protocol presented here allows for the assessment of chronic effects on mortality, growth, reproduction (fertility) and development (larval development to D-shaped stage, percentage of larval deformities). The test focuses on reproduction as an integrative endpoint. Changes in sex ratio and gamete quality compared to a control can be induced by several substances known to have an impact on reproduction. The test covers the assessment of hormonally active substances but is not exclusively sensitive to EDCs and is equally applicable to the detection of adverse effects on reproduction and development mediated via other modes of action.

A brief overview of the candidate protocol is presented below:

5 adult *Crassostrea gigas* (P generation) of each sex have to be obtained from an oyster hatchery. Adults should be maintained for a minimum of two weeks under laboratory conditions in the run-up to spawning. To induce spawning, oysters have to be conditioned by thermal stimulation (alternate placing of oysters in 18 °C and 28 °C sea water) and by adding small quantities of heat-killed ripe gametes. Alternatively oysters can be opened and gametes can be extracted by stripping via clean Pasteur pipettes. Eggs and sperm from each oyster are deposited in separate glass vessels. It is recommended to purify the egg suspension (removal of cell impurities) by filtering via a 100 µm sieve and checking subsamples for viable eggs (spherical in shape). Subsamples of viable eggs are pooled. Egg density should be adjusted to 10,000 eggs/mL. Before fertilization, sperm subsamples should be microscopically assessed for motility and (for removal of cell impurities) filtered by a 60 µm sieve. Subsamples of motile sperm are pooled.

After 15 minutes of settlement, sperm suspension is added to egg suspension and gently stirred with a glass rod. For successful fertilization, sperm density of the mixture should be between $10^5$-$10^7$ sperm/mL. Fertilization success is checked microscopically after a period of 4 hours (2-4 cell stage) before adding the embryo suspension (F0 generation) to a triplicate of 2 L test vessels. To provide for an almost equal concentration of embryos in each test vessel, the embryo suspension should be well mixed while extracting organisms via micropipette with an enlarged tip. Final embryo density should be 200 individuals/mL and may require an adjustment of the embryo suspension with stock sea water. Density of embryos can be assessed by taking a 1 mL aliquot from the embryo suspension and counting individuals in a Sedgwick-Rafter chamber (200 x magnification).

100 embryos (0.5 mL embryo suspension) are added to the test vessels containing 1 L test medium. The latter is dosed in advance with a given concentration of the test substance. One month post fertilization (pf) 50 individuals of developed juveniles (spat) are removed from the test vessels into 250 L glass aquaria and exposed for a further 9 months. According to Żbikowska et al. (2006) sexual maturity of the parental test organisms should be obtained at a shell length above 30 mm (7 months pf). However, as the species is known to be a protandric hermaphrodite the exposure must be extended to 10 months to also cover the period of transition to female sex. Individuals should be placed with the concave left valve down. The open and hinge-free parts of all oysters should be oriented in the same direction towards the incoming flow of the test solution. Dead individuals are removed immediately from the exposure systems. Survival rate and mean shell length (growth rate) of the P generation is determined and calculated at monthly intervals. After 10 months of exposure, adult and sexually mature individuals of the F0 generation are microscopically sexed and sex ratio of adults is determined.

Thereafter a series of cross-breeding experiments can be carried out with gametes of the exposed F0 generation. Gamete extraction and fertilization should be performed as mentioned before. The density of the resulting embryos (F1 generation) is adjusted to 200 individuals/mL. 0.5 ml of the F1 embryo suspension at the 2-4 cell stage (4 hours pf) is added to 30 mL test vessels equipped with the test medium and embryos are exposed for a further 48 hours. Number of replicate per test cross is 4. At the end of the exposure the F1 embryo development is stopped by adding 0.5 mL of 20 % formalin to the test medium. Effect concentrations are calculated for the following endpoints for each test run: larvae that accomplished

D-shape (ECx), normal larval development without deformities (ECx) and mortality ($LC_{50}$).

**Material and Methods**

Equipment

Test vessels and other apparatus used should ideally be made of glass or other materials chemically inert to the test chemical. All glassware must be washed thoroughly to remove any contaminant. Before washing, glassware should be sterilised by autoclaving. An overnight soak of all test equipment in a decontamination agent such as DECON$^{TM}$ 90 (5% DECON 90 in tap water) is necessary, the final rinse being in deionised distilled water.

The following equipment is required:

- 2000 mL glass beakers (for breeding and exposure of the F0 generation)
- 250 L glass aquaria for exposure of the F0 generation until adulthood
- 30-50 mL glass beakers (for F1 cross-breed experiments)
- glass pipettes
- oxygen meter
- pH meter
- thermometer
- refractrometer to check salinity/water conditions
- (stereo)microscope
- Sedgwick-Rafter chamber
- climate chambers or temperature regulated room with temperature indicator
- algal culturing facilities

Test medium

Artificial seawater should be used as test medium. The water should be prepared and stored in 500 litre aquaria. Before use it should be aerated for at least 24 hours and circulated by a water pump continuously to guarantee complete dissolution of the minerals and stripping of $Cl_2$.

Test conditions

Water parameters should be as follows:

- temperature 20 ± 1°C (for rearing of P and cross-breeding experiments)
- pH: should not vary more than 0.5 pH units
- oxygen concentration: > 6 mg/L
- oxygen saturation: > 60% throughout all tests (F0 and F1)
- salinity: > 12 ± 2‰

Initial test vessels for the F0 generation should contain 1 L of artificial seawater. Water should be circulated and aerated through glass pipettes (Pasteur pipettes). Dissolved oxygen content should be kept above 60%, however the test aquaria should be aerated as little as possible to avoid stripping of test chemicals. The water in each test aquarium should be completely renewed at least three times a week (preferably synchronized with feeding interval). Water quality variables (pH, dissolved oxygen content, temperature and total nitrite content) should be measured before the water renewal in all test beakers. Additional measurements of ammonium and nitrate should be done if necessary. Constant photoperiods of 12 hours light to 12 hours darkness should be maintained. Developed F0 spat should be transferred 1 month pf to 250 L glass aquaria equipped with artificial seawater. F0 individuals are exposed for a further 9 months until adulthood. Water quality conditions for the maturing F0 generation should be as mentioned above. For circulation of the test medium in 250 L aquaria, the installation of a water pump filter system is required. Test vessels for the F1 generation should contain 30 mL of artificial seawater. Water should be

gently aerated through glass pipettes (Pasteur pipettes).

Feeding of test animals

Oysters should preferably be fed daily but at least three times per week (at a two-days interval) with a mixed algal supply (*Isochrysis galbana*, *Pavlova lutheri*, *Chaetoceros mureli*) or suitable freeze-dried algal suspension ad libitum. Algal density (fresh cultures) in the exposure vessel at the start of feeding should be in the range of 20,000-50,000 cells/mL (Nice, 2005; Walne, 1970).

Test concentrations and replicates

Chemical stability analyses should be performed in the run-up to the test to check for stability of the test substance as degradability may influence renewal intervals of test media and substance. Usually the test substance will be added with every renewal of the exposure water.

At least 5 concentrations and a control group with four replicates should be tested in a geometric series with a factor between concentrations not exceeding 2.2. In the run-up to the definitive test the performance of range finding studies will help to select appropriate test concentrations.

If a solubilizer has been applied, a solvent control with the same amount of solvent as in the treatments should be used. The amount of solvent in test concentrations should be as small as possible, not exceeding a maximum concentration of 0.01% (100 µl/l or 100 mg/l). Solvent and concentration selection depends on the chemical constitution of the test substance and sensitivity of test organisms and is usually determined in a precursor study. Further information on appropriate organic solvents may also be obtained from Jarosz and Weltje (2008).

**Biological methods**

The test aquaria should be checked at least three times per week for abnormal test conditions (e.g. fungi infections) or behaviour of test organisms (for example shell gaping).

Adult oysters (P generation) should be taken from either a healthy breeding stock maintained in the lab, commercial harvesters or collected from unpolluted field populations free from parasitism. They must not show any sign of stress which is normally revealed by excessive mucus production, lack of feeding, shell gaping, poor shell closure in response to nudging, or enhanced mortality. Test organisms should be maintained in culture conditions (light, temperature, medium, salinity and feeding) similar to those of a laboratory breeding stock/hatchery or field-grown population.

Mean oyster shell length (maximum distance between anterior and posterior margin parallel with hinge axis) of the F0 generation should be monitored at the beginning of the test and later at monthly intervals throughout the duration of the experiment. Shell length of F0 replicates (first month pf) should be in the same order of magnitude and must not show statistically significant differences. Therefore 50 F0 oysters of comparable size have to be allocated randomly to each test vessel/aquarium containing the exposure water. At the beginning of the experiment, shell length of young oysters should be measured via stereomicroscopical analyses with an ocular micrometer. At later dates, shell length can be determined by digital callipers.

For sex ratio assessment, gonad punctures are performed by drawing a Pasteur pipette five times along the length of the organ. Sex is determined via microscopical investigation and the percentage of male, female and hermaphrodite adult oysters is calculated for each replicate. F0 gamete samples are further investigated to determine the mean percentage of oysters with motile sperm and viable eggs for each test concentration (fertility assessment). Unfit eggs resemble a teardrop shape whereas viable eggs are spherical. Sperm motility is evaluated after loading chambered slides with gamete samples suspended in a drop of seawater for 10 minutes. This time interval should be sufficient for sperm (in contact to seawater) to become motile. According to Nice (2005), oyster sperm remains motile upon contact to seawater for up to 5 hours. The mean percentage of oysters with motile sperm and viable eggs is identified.

Evaluation of the cross-breeding experiments resulting in F1 larvae should include survival expressed as larval density/mL compared to the control and/or $LC_{50}$ Furthermore the $EC_x$ values for larvae that achieved D-shape and the percentage of larval deformities (according to His et al., 1997, larvae with convex hinge, indented shell margin, incomplete shell and protruding mantle) compared to normal larvae of the controls is determined.

**Data and reporting**

Validity criteria

Oxygen concentration in the water should not be below 60% of air saturation value at test temperature before water renewal.

The pH of the water should not vary more than 0.5 pH units.

The concentration of the test substance should be measured to ensure that the concentration has been maintained over the test period.

Mortality of adult P control oysters should not exceed 20%.

Fertilization success of appropriately conditioned and maintained control oysters should result in at least 70% normal embryos.

Appropriate positive controls have to be applied to demonstrate the responsiveness of test animals to EDCs.

Treatment of results

When planning the test, it should be taken into consideration whether the aim is to calculate a NOEC/LOEC (by use of ANOVA or comparable nonparametric tests) or ECx values (by use of (non-)linear regression). The demand for replicates is higher if the ANOVA statistic is used whereas the (non-)linear regression generally demands more concentrations.

Endpoints to be statistically evaluated are mortality, shell length (growth), sex ratio, gamete motility (fertility), larval survival, development rate and developmental aberrations. Data are recorded e.g. in a spreadsheet, and mean and variability parameters such as standard deviation or standard error of the mean are calculated.

ECx (effect concentration)

ECx values are calculated using an appropriate statistical model (e.g. logistic or Weibull function). An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation. To compute the $EC_{50}$ or any other ECx, the individual replicates should be used as input for the regression analysis.

NOEC/LOEC (No Observed Effect Concentration/Lowest Observed Effect Concentration)

If a statistical analysis is intended to determine a NOEC/LOEC value, a one-way analysis of variance (ANOVA) and a multiple comparison procedure is recommended. The latter allows the definition of a treatment concentration that characterizes significant differences for reproduction/development parameters compared to the control. One-way analysis of variance is based on the assumptions that the observations are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using the ANOVA. If data sets do not comply with these requirements the normality and the homogeneity of variances can be increased by the following transformation of data before testing (cf. section 6.5.4 for details):

- quantal responses expressed as a percentage (e.g. survival or mortality, proportion of females or males) should be arcsine-square root transformed,

- counts (e.g. numbers of produced eggs and clutches or number of embryos in the brood pouch) should be square root transformed,
- continuous data (e.g. growth or weight measures) should be transformed to the natural logarithm.

Following transformation, data sets have to be tested again for normal distribution and homogeneity of variances. Gaussian distribution can be verified e.g. by the Kolmogorov-Smirnow-T normality test, whereas Bartletts's Test test represents a method for checking homogeneity of variance. In case data do not meet the assumptions for ANOVA, non-parametric procedures such as Kruskal-Wallis-Test should be used. For the analysis of mortality (quantal data) the Fisher's exact test is recommended if the mortality of the individuals is independent. If individuals are living in the same test vessel, this assumption is violated. If data follow a monotone concentration response, a step-down trend test such as Cochran-Armitage and Jonkheer should be used to analyse quantal responses.

**Reporting requirements**

Laboratory reports should include the following information:

Testing site

- Identification of the laboratory, dates of testing and key personnel involved

Test substance

- chemical identification of the substance (name, structural formula, CAS number, etc.) including purity, manufacturer, lot or batch number
- relevant physicochemical properties (e.g. water solubility, vapour pressure)
- analytical method for quantification of the test substance where appropriate
- appropriate negative and positive control

Test species

- scientific name, source and culture conditions

Test conditions

- test procedure applied including photoperiod and light intensity, test design (e.g. test concentration used, number of replicates, number of oyster larvae per replicate)
- method of test substance pre-treatment, stock solution preparation and substance delivery system
- nominal test concentrations, details on sampling for chemical analysis of the test concentrations and the analytical methods by which the substance concentrations were analysed (including recovery and detection limit)
- information on the test conditions regarding pH, salinity, hardness, conductivity, temperature and dissolved oxygen concentration and saturation, nitrate, nitrite and ammonium concentrations
- information on feeding (e.g. source and type of food, quantity, feeding interval).
- compliance with validity criteria

Results

- references to chemical and statistical procedures (including assessment of the assumptions on which they are based)
- tabular and graphical (when appropriate) presentation of all measured and calculated endpoints
- tabular presentation of measured test concentrations including quality control samples
- NOEC/LOEC (including mean values, standard errors, error degrees of freedom and the least significant difference, or standard error of the difference) or ECx values (including the mathematical equation of the model used to estimate Ecx values, the slope of the dose response,

confidence limits around the slope, assessment of goodness-of-fit and if possible assessment of appropriateness of the model applied) should be reported and p-values should be specified

- any deviation from the protocol accompanied by a discussion on possible impact on the study results

**Recommended Protocols – data gaps and validation requirements**

## 1.24 Data gaps and research requirements

In general terms, molluscan reproductive rates are very sensitive to environmental conditions. Particularly important variables are temperature, daylength, season, population density and food quality. The influences of all these factors on the candidate species are known to greater or lesser extents, and all except season can, of course, be controlled in the laboratory. In the case of season, it has been known for many years that some molluscs will not reproduce if brought into the laboratory during the autumn or winter (e.g. Precht, 1936), so it is strongly recommended that all experiments are conducted with artificially reared animals that have been held for their entire lives under conditions of controlled temperature and daylength. However, even under such controlled conditions without external triggering, molluscs may retain a seasonal pattern in reproduction. This has to be taken into account when interpreting the results of tests with EDCs.

In the case of the other variables, although we know they are important controlling factors, and the draft protocols accordingly specify broadly suitable test conditions, we probably need to conduct further research in order to more precisely specify acceptable and optimally sensitive ranges of test conditions.

Both long daylength and increased temperatures are key triggers for reproduction in gastropods (e.g. Geraerts, 1986; Bohlken and Joose, 1992; Costil and Daguzan, 1995), and the availability of sufficient nutritious food is also critical for good gamete quality and successful reproduction (e.g. Dogterom *et al*., 1985). Increased crowding or reduced water quality may also stimulate reproduction in some species (the so-called drying pond effect) (Dillon, 2000), or reduce it in others (Aufderheide *et al*. 2006), and if this is accompanied by poor food quality, insufficient energy may be devoted to egg development, leading to small or weakened offspring.

### 1.24.1 The freshwater mudsnail (*Potamopyrgus antipodarum*)

Substantial ecotoxicological research has been conducted with this species in partial life cycle tests, and suitable test conditions have been proposed by Duft *et al*. (2007) and in section 0of this DRP. However, these test conditions, including snail density, temperature, daylength and feeding rate, were mainly chosen in order to guarantee appropriate reproduction according to an annual reproductive cycle. In order to provide more knowledge about optimal sensitivity and acceptable variability of test conditions, it would be desirable to conduct a series of experiments with a standard test chemical such as EE2, presented in solution. A particularly important issue concerns the phenomenon of increased fecundity in response to estrogens. This response seems to be highly dependent on the temperature regime, and it is possible that sensitivity can be increased by manipulating test conditions (e.g. by reducing the temperature) such that control reproductive rate is sub-maximal (but still within the normal range).

Given the substantial experience already available with this species/protocol, it is not considered essential to conduct the research outlined above before validation commences. Such work could more efficiently be conducted in parallel with validation.

### 1.24.2 The pond snail (*Lymnaea stagnalis*)

This species has also been widely used for ecotoxicological testing in both partial and full life cycle experiments, so suitable test conditions are reasonably well established. However, the test conditions specified in section 0 have been derived by combining the information in a number of publications (Bandow 2009, Bandow and Weltje 2008, Czech *et al*. 2001, Coutellec and Lagadic 2006, Coutellec *et al*. 2008, De Schamphelaere *et al*. 2008, Ducrot *et al*. 2008 a, b; Leung *et al*. 2007, Présing 1993, Ter Maat *et al*. 2007, Weltje *et al*. 2003), and as with *P. antipodarum*, it will be necessary to validate the proposed test procedures with a range of EDCs. One variable that is not relevant here is sediment, because *L. stagnalis* is tested in water alone, but the other variables which are vital driving factors in gastropod reproduction (temperature, daylength, feeding rate and population density) would probably benefit from further optimisation, again with standard test chemicals.

Given the fact that this protocol has been composed of various methods published in the literature, rather than it having been fully tested as it stands, it would probably be desirable to conduct this optimisation research before validation commences.

### 1.24.3 The Pacific oyster (*Crassostrea gigas*)

Although this species is widely reared in aquaculture systems, so optimal conditions for growth and reproduction are well known, it has been used relatively infrequently for life cycle tests with EDCs. The test protocol proposed in section 7 has been composed from methods used by several authors (Geffard *et al*. 2002, 2003, His *et al*. 1997, Nice *et al*. 2000, 2003, Nice 2005, Thain 1992, Waldock and Thain, 1983), but it appears likely that its sensitivity will require optimisation with at least one standard test substance. One factor of particular interest with this species is the influence of genetic variations on sensitivity to EDCs. This is because the test is started with gametes harvested from adults obtained from a hatchery, and there are probably several strains with unknown variations in susceptibility to chemicals. Strain-variations in sensitivity may also be a factor for the other two species, but as these can be successfully bred in the laboratory, it would be possible to ensure that all test laboratories use the same strain. A related factor is the quality of the parent gametes, which depends to a large extent on the type and amount of food given to the parents. It is vital that parental (P) feeding is optimal, because poor quality food can cause abnormalities in the resulting F0 embryos, and presumably can also impair the ability of the F0 adults to reproduce. It is possible that the sensitivity of *C. gigas* to chemicals could be enhanced by manipulating parental feeding, but little is known about this subject.

The behaviour of this protocol is the least well understood of the three, and it certainly will require optimisation before a validation programme can be recommended.

### 1.24.4 Other species

Earlier sections in this DRP mention several other molluscan species which show possible promise for use in standardised test protocols. Some examples are the gastropods *Planorbarius corneus* and *Crepidula fornicata*, and the bivalve *Ostrea edulis*, all of which are easily bred in the laboratory but for which there is very little testing experience to date. Furthermore, some species (e.g. *Hydrobia ulvae and Viviparus viviparus*) also have desirable properties such as sensitivity to EDCs, but are currently hard or impossible to culture or breed in the laboratory. Concerning *Hydrobia* spp., it might be worth considering *H. ventrosa* and *H. neglecta*, neither of which have pelagic larvae so they may therefore be easier to culture than *H. ulvae*. The tropical species *Biomphalaria glabrata* has also been extensively used in toxicity tests (mainly with non-EDCs) and could be worthy of further investigation. It may consequently be considered desirable to conduct developmental research with some of these other species in case back-ups are needed if any of the first-choice species eventually prove to be unsuitable.

### 1.25 Validation requirements

Any programme of validation of mollusc partial- and full-life cycle tests will be required to conform to the principles set out in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (OECD, 2005). These principles are set out below.

The first significant principle concerns intra-laboratory repeatability. Good data on this subject already exist for the *P. antipodarum* protocol, but there appear to be less published repeatability data available for *L. stagnalis*, and almost none for *C. gigas*. It would clearly be desirable to obtain such data before proceeding further, but that of course should not be done until the respective protocols have been optimised.

The next important principle, and probably the most crucial one, is inter-laboratory reproducibility. This establishes whether the protocols can be conducted adequately in different laboratories with a range of typical test chemicals, in order to obtain results that are acceptably consistent. Suitable test chemicals might include strong and weak estrogens, strong and weak androgens, an aromatase inhibitor, and various organotin compounds. It is also important to show in at least one laboratory that the protocols do not respond to an agreed set of negative chemicals (i.e. those which are known to have no impact on mollusc reproduction). Such data are essentially unavailable at present for the three candidate mollusc protocols.

Given the amount of research work required before the *C. gigas* (and to a lesser extent the *L. stagnalis*) protocols can be optimised, it would probably be appropriate to begin any validation programme with the *P. antipodarum* partial life cycle protocol.

The following test validation principles are set out in OECD (2005):

a) **The rationale for the test method should be available.**
This should include a clear statement of the scientific basis, regulatory purpose and need for the test.

b) **The relationship between the test method's endpoint(s) and the (biological) phenomenon of interest should be described.**
This should include a reference to scientific relevance of the effect(s) measured by the test method in terms of their mechanistic (biological) or empirical (correlative) relationship to the specific type of effect/toxicity of interest. Although the relationship may be mechanistic or correlative, test methods with biological relevance to the effect/toxicity being evaluated are preferred.

c) **A detailed protocol for the test method should be available.**
The protocol should be sufficiently detailed and should include, *e.g.,* a description of the materials needed, such as specific cell types or construct or animal species that could be used for the test (if applicable), a description of what is measured and how it is measured, a description of how data will be analysed, decision criteria for evaluation of data and what are the criteria for acceptable test performance.

d) **The intra-, and inter-laboratory reproducibility of the test method should be demonstrated.**
Data should be available revealing the level of reproducibility and variability within and among laboratories over time. The degree to which biological variability affects the test method reproducibility should be addressed.

e) **Demonstration of the test method's performance should be based on the testing of reference chemicals representative of the types of substances for which the test method will be used.**
A sufficient number of the reference chemicals should have been tested under code to exclude bias (see paragraphs on “Coding and Distribution of Test Samples”).

f) **The performance of the test method should have been evaluated in relation to relevant information**

**from the species of concern, and existing relevant toxicity testing data.**

In the case of a substitute test method adequate data should be available to permit a reliable analysis of the performance and comparability of the proposed substitute test method with that of the test it is designed to replace.

g) **Ideally, all data supporting the validity of a test method should have been obtained in accordance with the principles of GLP.**

Aspects of data collection not performed according to GLP should be clearly identified and their potential impact on the validation status of the test method should be indicated.

h) **All data supporting the assessment of the validity of the test method should be available for expert review.**

The detailed test method protocol should be readily available and in the public domain. The data supporting the validity of the test method should be organised and easily accessible to allow for independent review(s), as appropriate. The test method description should be sufficiently detailed to permit an independent laboratory to follow the procedures and generate equivalent data. Benchmarks should be available by which an independent laboratory can itself assess its proper adherence to the protocol.

**Culture and handling of recommended species**

## 1.26 Methods

*Potamopyrgus antipodarum* and *Lymnaea stagnalis* are both freshwater species and easy to culture in the laboratory. In contrast, the culture of the marine bivalve *Crassostrea gigas* is more challenging and ideally requires access to natural sea water to minimise maintenance and operating expenses. It has to be guaranteed that good quality seawater is available the whole year round. Depending on the natural surroundings, salinity may vary widely (e.g. estuarine areas with brackish water, areas with frequent periods of heavy rainfall). Nevertheless it would be desirable that water quality parameters for OECD purposes are comparable worldwide if not completely standardized. In any case, the selection of proper sites for *Crassostrea* culture is important. Negative (toxic) impacts of algal and bacterial blooms must be prevented and pollution originating from agricultural land-use, and municipal and industrial sewage treatment plant effluents has to be excluded. Seawater supply from areas close to ports and marinas should also be regarded as impermissible.

Generally, technical staff should be trained in appropriate breeding and handling methods regarding all candidate species. To avoid contamination of culture systems with personal care products (i.e. UV-screens, body lotions, insect repellents - and potentially tobacco smoke residues) staff should comply with defined hygiene regulations. Tanks, glassware and other equipment used to establish and maintain breeding of test species have to be cleaned and sanitised prior to use. Preference should be given to chemical-free methods and application of non-toxic detergents without harsh chemicals. Full ingredient disclosure should be provided as some components of cleansing agents are suspected to have a negative impact on reproduction and/or development of aquatic test species.

Breeding facilities should preferably be fabricated from and equipped with inert materials like glass and stainless steel to avoid leaching of e.g. plasticizers, heavy metals and surface protectants. Copper-based water supply systems (especially when they are newly installed) have to be handled with care and may require additional filter systems as the metal is known to be acutely toxic to molluscs at concentrations in the µg/L range (e.g. see Mischke et al. 2005, Soucek and Noblet 1997).

### 1.26.1 The freshwater mudsnail (*Potamopyrgus antipodarum*)

**Equipment**

The following equipment is required:

- culturing tanks (10 litres, glass or stainless steel)
- aquaria (≥ 50 L) to prepare reconstituted (synthetic) water
- aquarium power filters (equipped with ceramic rings, biological filter gravel and substrates, cockle shell and coral fragments) for aquaria of the brood stock. Note: Power filter and any other plastic material has to be checked prior to use to ensure no leaching of plasticizers and other EDCs.
- air pumps
- flexible air tubes
- glass pipettes
- oxygen meter

- pH meter
- conductivity meter
- test kits for ammonium, nitrite and nitrate measurements in water
- stereomicroscope
- cold light source
- dissecting dish & dissecting instruments
- climate chambers or temperature regulated room with temperature indicator

**Food and chemicals for water conditioning**

The following compounds and products are needed:

- TetraPhyll® or equivalent proprietary feed
- Sodium hydrogen carbonate ($NaHCO_3$)
- Calcium carbonate ($CaCO_3$)
- Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
- Tropic Marin® or equivalent proprietary sea salt

**Culture medium and condition**

For the breeding of *Potamopyrgus* reconstituted water is prepared in storage tanks ≥ 50 L. Therefore 12.5 g Tropic Marin® sea salt and 7.5 g Sodium hydrogen carbonate ($NaHCO_3$) are dissolved in 50 L deionised water. Before use water has to be aerated for at least 24 hours and circulated continuously by a water pump to guarantee complete dissolution of all ingredients.

Water quality parameters should be as follows:

- Temperature: 16 ± 1°C
- pH: 8.0 ± 0.5
- Oxygen concentration: > 6 mg/l
- Oxygen saturation: > 60%
- Conductivity: 770 ± 100 µS/cm

Constant photoperiods of 16 hours light to 8 hours darkness should be maintained.

**Population density**

The population density must not be higher than 150 snails per litre.

**Food and feeding**

Feeding should be done daily with finely ground commercial feed such as TetraPhyll® ad libitum (approx. 0.25 mg per animal). A cuttlebone in every breeding aquarium may support shell growth.

**Cleaning and care**

Breeding aquaria should be maintained without bottom substrate. The breeding medium should be partially renewed every two weeks. For this purpose one third of the water has to be replaced by fresh reconstituted water from the storage aquarium. It is important not to remove the whole medium, but approximately one-third of the water together with detritus and other deposits, as the established nitrogen-fixing bacteria must not be removed. Aquaria and filter pumps have to be cleaned thoroughly but at infrequent intervals (approximately every 6 weeks) when filter material is blocked and water flow of the pumps declines. Depending on biofouling, filter materials have to be cleaned or replaced by up to 70% of their volume, as extensive exchange will lead to enhanced ammonia and pH values (for ammonia effects on *Potamopyrgus antipodarum* see Alonso and Camargo, 2009). Water quality parameters (e.g. pH, dissolved oxygen,

conductivity, temperature and total nitrite) should be measured before water renewal in all breeding aquaria. Additional measurements of ammonium and nitrate should be made if necessary (e.g. if the aquaria are newly installed). Measurement of water quality parameters in the breeding aquaria must be done with thoroughly cleaned electrodes. They should also be rinsed clean before they are used in the next aquarium to prevent a potential transfer of diseases or pathogens. Aquaria should be checked daily for abnormal breeding conditions (e.g. bacterial or fungal growth) or behaviour of test organisms (for example avoidance of water) at the same time as feeding.

**Monthly recording of embryo numbers**

Fecundity should be determined at monthly intervals to reveal the annual reproductive cycle under "controlled" conditions (without exposure). Therefore, 20 sexually mature individuals (shell height normally between 3.5 and 4.0 mm) should be narcotised for 45-90 minutes in a solution of 2.5% magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$) dissolved in deionised or distilled water prior to dissection.

Shell and aperture height of snails should be measured via stereomicroscopical analyses with an ocular micrometer. Thereafter shells should be cracked with a small vice or combination pliers. Soft bodies are dissected by removing the shell with pointed forceps in a solution of magnesium chloride hexahydrate (2.5%). The brood pouch of the snails should be opened carefully with a dissecting needle, embryos removed and counted, distinguishing shelled and unshelled embryos.

Endpoints to be evaluated statistically are the total number of embryos and the number of unshelled embryos per female. Data are recorded e.g. in a spreadsheet. The mean, and variability parameters such as standard deviation or standard error of the mean, are calculated for the shell height, aperture height, number of embryos with shell, number of embryos without shell, and the total number of embryos.

**Parasitism, algal growth, diseases and mortality**

For many years European populations of *Potamopyrgus antipodarum* were regarded to be nearly free from parasites. According to Morley (2008) this opinion mainly resulted from a lack of scientific studies investigating the actual parasite infestation of the New Zealand mudsnail in Europe. The author points out that in its natural environment the snail is known to act as intermediate host for a number of trematodes. Although there is a lack of information on parasite prevalence and incidence in European *Potamopyrgus* populations (Morley 2008), some New Zealand trematode species (echinostomes, microcercous metacercariae, tetracotyle metacercariae) have been described in U.K. and French populations (e.g. Beverly-Burton 1972, Bisset 1977, Gérard et al. 2003). When building up a new breeding stock with feral *Potamopyrgus* snails this should be appropriately addressed. Morley (2008) recommends examination of the parasitism status of a subsample of 100 individuals before proceeding with culturing or ecotoxicological studies. Normally populations affected with parasitic metazoans (e.g. tremotodes, cestodes) can be identified by stereomicroscopic investigation (preferably of the digestive tract) and microscopical specimen preparation. Protistan infestation requires microscopic analysis including full-length sagittal sections throughout the animal body. In case of platyhelminth infection it might be sufficient to exclude the parental generation from the experiments, as the successful distribution of the parasite is intrinsically tied to an alternation of hosts. However in other cases (diseases caused by protistans) this procedure may not be sufficient and the breeding of a parasite-free population will take much more time and effort if possible at all. Therefore it is recommended to exclude such populations from breeding

Depending on light intensity, heavy algal growth may occur on the shell of snails. Under natural conditions algal cover is removed by sediment abrasion. If possible, algae should be manually removed with micro-scissors as intensive algal cover poses a stress-factor to the organisms. Ideally algal growth should be minimised by arranging optimal exposure to light. Artificial lighting may require the shadowing or exclusion of natural sunlight. Given the fact that algal growth is not completely controllable in this way,

affected snails have to be removed from the brood.

If unusual behaviour (e.g. lethargy with drooped soft body, unresponsiveness to mechanical irritation of the soft body) indicating a bad health state is observed, these snails have to be removed. If such indications are frequently observed in an aquarium, the snails in this aquarium must be removed from the breeding colony and the aquarium with all equipment must be thoroughly cleaned and disinfected to prevent spread of diseases.

Dead snails must be frequently removed from the breeding group. If there is a breeding aquarium with enhanced mortality (according to Council Directive 95/70/EC sudden mortality affecting approximately 15% of stocks and occurring over a short period between two inspections (confirmed within 15 days) (EU 1995) all snails must be excluded from the breeding colony and humanely killed by overdosing of narcotics (e.g. 24 hours exposure to 2.5% $MgCl_2$ solution). Afterwards the aquaria and all equipment must be cleaned and disinfected thoroughly.

### 1.26.2 The pond snail (*Lymnaea stagnalis*)

**Equipment**

The following equipment is required:

- culturing tanks (50 litres; glass or stainless steel)
- preparation/storage tanks (≥ 100 L) to hold reconstituted (synthetic) water
- aquarium power filters (equipped with ceramic rings, biological filter gravel and substrates, cockle shell and coral fragments). Note: the power filter and any other plastic material has to be checked prior to use to ensure no leaching of plasticizers and other EDCs.
- air pumps
- flexible air tubes
- glass pipettes
- oxygen meter
- pH meter
- conductivity meter
- test kits for ammonium, nitrite and nitrate measurements in water
- stereomicroscope
- cold light source
- climate chambers or temperature regulated room with temperature indicator

**Food and chemicals for water conditioning**

The following compounds and products are needed:

- TetraPhyll® or equivalent proprietary feed
- Sodium hydrogen carbonate ($NaHCO_3$)
- Calcium carbonate ($CaCO_3$)
- Magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$)
- Tropic Marin® or equivalent proprietary sea salt

**Culture medium and condition**

For the breeding of *Lymnaea* reconstituted water is prepared in storage tanks ≥ 100 L. Therefore 25 g Tropic Marin® sea salt and 15 g Sodium hydrogen carbonate ($NaHCO_3$) are dissolved in 100 L deionised water. Before use water has to be aerated for at least 24 hours and circulated continuously by a water pump to guarantee complete dissolution of all ingredients. Water parameters should be as follows:

- Temperature: 20 ± 1°C
- pH: 8.0 ± 0.5
- Oxygen concentration: > 6 mg/l
- Oxygen saturation: > 60%
- Conductivity: 770 ± 100 µS/cm

Constant photoperiods of 16 hours light to 8 hours darkness should be maintained.

**Population density**

The population density must not be higher than 5 snails per litre.

**Food and feeding**

Feeding should be done daily with finely ground commercial feed such as TetraPhyll® ad libitum. Alternatively, fresh lettuce can be offered to the snails, but it should originate from organic farming. A cuttlebone in every breeding aquarium may support shell growth.

**Cleaning and care**

Breeding aquaria should be maintained without bottom substrate. It is important to notice that handling of snails during cleaning procedures should be done with utmost care. Besides air, stressed animals eject hemolymph when disturbed or removed from aquaria. The snails need 24 hours to recover from this stress reaction. To avoid stress, only free floating snails should be used or otherwise removed from the glass wall with the help of a brush that is carefully inserted between body and sub font. The breeding medium should be partially renewed every week. For this purpose half of the water has to be replaced by fresh reconstituted water from the storage aquarium three times a week. It is important not to remove the whole medium, but approximately one-third of the water together with detritus and other deposits, as the established nitrogen-fixing bacteria must not be removed. Aquaria and filter pumps have to be cleaned thoroughly but at infrequent intervals (approximately every 6 weeks) when filter material is blocked and water flow of the pumps declines. Depending on biofouling, filter materials have to be cleaned or replaced by up to 70% of their volume, as extensive exchange will lead to enhanced ammonia and pH values. Water quality parameters (e.g. pH, dissolved oxygen, conductivity, temperature and total nitrite) should be measured before the water renewal in all breeding aquaria. Additional measurements of ammonium and nitrate should be made if necessary (e.g. if the aquaria are newly installed). Measurement of water parameters in the breeding aquaria must be done with thoroughly cleaned electrodes. They should also be rinsed clean before they are used in the next aquarium to prevent a potential transfer of diseases or pathogens. Aquaria should be checked daily for abnormal breeding conditions (e.g. bacterial or fungal growth) or behaviour of test organisms (for example avoidance of water) at the same time as feeding.

**Monthly recording of reproductive success**

Reproductive success (eggs, clutches and eggs/clutch) of 20 snails (5 replicates, 4 snails per replicate) should be analysed once per month over a period of three days to determine the annual reproductive cycle under "controlled" conditions (without exposure). Shell and aperture height of snails are measured using digital callipers. Clutches produced in the three day period are counted and removed by razor blades from the tank surface. Total number of eggs and the egg number per clutch are counted for all replicates.

Endpoints to be evaluated statistically are the total number of eggs and clutches, egg number per clutch and egg number per parent. Data are recorded e.g. in a spreadsheet. The mean, and variability parameters such as standard deviation or standard error of the mean, are calculated for each of the endpoints.

**Parasitism, diseases and mortality**

Depending on season and location, parasite infestation rates for wild-caught *Lymnaea stagnalis* have been reported to be rather high. Loy and Haas (2001) examined more than 40,000 *L. stagnalis* from 174 pond systems in South Germany during 1980-2000 and identified 18 cercarian species with an overall prevalence of 44.9%. Żbikowska et al. (2006) demonstrated that more than 50% of *Lymnaea* specimens originating from the nearshore zone of the Jeziorak Lake (Northern Poland) were parasitised by different trematode species (*Plagiorchis elegans, Diplostomum pseudospathaceum, Notocotylus attenuatus, Trichobilharzia ocellata, Echinostoma revolutum, Echinoparyphium aconiatum*). When building up a new breeding colony with feral pond snails, potential parasitism should therefore be considered. In any case it requires the quarantining of animals to interrupt the transmission of parasitic generations. Normally populations affected with parasitic metazoans (e.g. tremotodes, cestodes) can be identified by stereomicroscopic investigation (preferably of the digestive tract) and microscopical specimen preparation. Protistan infestation in any case requires microscopic analysis including full-length sagittal sections throughout the animal body. In case of platyhelminth infection it might be sufficient to exclude the parental generation from the experiments, as the successful distribution of the parasite is intrinsically tied to an alternation of hosts. However in other cases (diseases caused by protistans) this procedure may not be sufficient and the breeding of a parasite-free population will take much more time and effort if possible at all. Therefore it is recommended to exclude such populations from breeding. Unusual behaviour associated with lethargy and unresponsiveness to mechanical irritation and a drooped soft body indicate a bad health state. These snails have to be removed from the breeding colony. If such indications are frequently observed in an aquarium, all snails must be removed from the breeding group. Afterwards, affected aquaria including equipment must be thoroughly cleaned and disinfected to prevent the spreading of diseases.

Dead snails must be frequently removed from the breeding colony. If there is a breeding aquarium with enhanced mortality, (according to Council Directive 95/70/EC sudden mortality affecting approximately 15% of stocks and occurring over a short period between two inspections (confirmed within 15 days) (EU 1995) all snails must be excluded from the breeding colony and humanely killed by overdosing of narcotics (e.g. 24 hours exposure to 2.5% MgCl2 solution). Afterwards the aquaria and all equipment must be cleaned and disinfected thoroughly.

### 1.26.3 The Pacific oyster (*Crassostrea gigas*)

**Equipment**

The following equipment is required:

- culturing tanks (100 litres; glass or stainless steel)
- preparation/storage tanks (≥ 250 L) to store seawater
- aquarium power filters (equipped with ceramic rings, biological filter gravel and substrates, cockle shell and coral fragments). Note: the power filter and any other plastic material has to be checked prior to use to ensure no leaching of plasticizers and other EDCs.
- air pumps
- flexible air tubes
- glass pipettes
- oxygen meter
- refractometer to check salinity
- pH meter
- test kits for ammonium, nitrite and nitrate measurements in water
- climate chambers or temperature regulated room with temperature indicator
- algal culturing facilities

**Chemicals for water conditioning**

The need for a supply of high quality seawater was previously discussed. If appropriate and unpolluted natural seawater is not available artificial seawater should be used as culture medium. Deionised tap water should be salinated with Tropic Marin® or equivalent proprietary sea salt and stored in 250 litre aquaria. Before use it should be aerated for at least 24 hours and circulated by a water pump continuously to guarantee complete dissolution of the minerals and stripping of $Cl_2$ or other tap water disinfectants.

Water parameters should be as follows:

- Temperature 20-24 ± 2°C (with fixed test temperature ± 1°C)
- pH 7.5 ±0.5 pH units
- oxygen concentration > 6 mg/L
- oxygen saturation > 60%
- salinity 32 ± 2‰

Constant photoperiods of 12 hours light to 12 hours darkness should be maintained.

**Population density**

The population density of adult broodstock (ideally obtained from commercial mariculture facilities) must not be higher than 20 individuals per 100 litres.

**Food and feeding**

Feeding should be done twice daily with a mixed algal supply (density of 20,000 cells/mL) of *Isochrysis galbana, Pavlova lutheri* and *Chaetocerus muelleri*.

A temperature regulated room (15 to 18ºC ) is required to maintain stock cultures of algae equipped with cool running fluorescent lights, compressed air and carbon dioxide supply (for detailed information please see Andersen 2005).

**Cleaning and care**

Culture aquaria should be maintained without bottom substrate. In semi-static systems (without flow-through of natural seawater) the breeding medium should be partially renewed every two weeks. For this purpose one third of the water has to be replaced by fresh reconstituted water from the storage aquarium. It is important not to remove the whole medium but approximately one-third of the water together with detritus and other deposits as the established nitrogen-fixing bacteria must not be removed. In flow-through systems with natural seawater supply aquaria should be cleansed of algae and detritus every 14 days.

In semi-static systems the circulation of the ambient water in the culture aquaria is maintained by water pump filter systems. Aquaria and filter pumps have to be cleaned thoroughly but at infrequent intervals (approximately every 6 weeks) when filter material is blocked and water flow of the pumps declines. Depending on biofouling, filter materials have to be cleaned or replaced by up to 70% of their volume, as extensive exchange will lead to enhanced ammonia values. Water quality parameters (pH, dissolved oxygen, salinity, temperature and total nitrite) should be measured before the water renewal in all breeding aquaria. Additional measurements of ammonium and nitrate should be made if necessary (e.g. if the aquaria are newly installed). Measurement of water parameters in the breeding aquaria must be done with thoroughly cleaned electrodes. They should also be rinsed clean before they are used in the next aquarium to prevent a potential transfer of diseases or pathogens. Aquaria should be checked daily for abnormal breeding conditions (e.g. bacterial or fungal growth) or behaviour of test organisms (e.g. permanent valve closure) at the same time as feeding.

**Monthly recording of gametogenic cycle**

In temperate regions *Crassostrea gigas* is known to maintain an annual reproductive cycle. Under natural conditions the spawning season and reproduction maximum occur during summer time (May - August in the northern hemisphere) and the sexual repose phase is during autumn and winter (Dinamani, 1987; Perdue 1982). Nevertheless, generation intervals have been described to be shortened artificially for several bivalve species by modulation of temperature, food and photoperiod parameters (Chávez-Villalba et al. 2001 and 2002, Matias et al. 2008, Martinez et al. 2000, Ruiz et al. 1992). Fabioux et al. (2005) maintained oysters under natural, accelerated and winter conditions and observed that "artificial spring" conditions in their laboratory experiments resulted in two complete gametogenic cycles per year. If it is not intended to purchase adult oysters from a commercial breeder but to rear them from the juvenile stage, it is recommended to run two shortened photoperiod and temperature cycles per year, according to Fabioux et al. (2005).

Qualitative and quantitative analysis of the reproductive cycle and sex ratio should be carried out once per month via histological investigation and subsequent image analysis (see Fabioux et al. 2005). Endpoints to be evaluated statistically are sex ratio and development of the gonad area (gametogenic cycle). Data are recorded e.g. in a spreadsheet. The mean and variability parameters such as standard deviation or standard error of the mean are calculated.

**Parasitism, diseases and mortality**

*Crassostrea gigas* is known to suffer from many diseases including parasitic (perkinsosis, haplosoridiosis, microcytosis), bacterial (*Vibrio* sp., juvenile oyster disease, "summer mortality") and viral (herpes-type virus disease) infections. The onset of the diseases is often linked to physiological stress in warm water after spawning, high population density and poor breeding conditions (for details see Elston, 1993, Helm 2006, ICES 2004, Renault and Novoa 2004).

Herpes-type virus infected larvae exhibit e.g. velar aberrations and therefore are no longer able to feed and swim. The disease spreads rapidly among the larvae and mortalities between 80 and 100% occur within a short time (6-12 days). Generally, inhibition of shell growth, cupping of the lower valve and loss of the growing edge of the upper valve, secretion of a conchiolin layer on both valves surrounding the soft tissue, green to yellow coloured pustules, abscess-like lesions of the soft body and tissue necrosis are indicative of bad health status (ICES 2004).If such indications are frequently observed, oysters in the respective aquaria must be removed from the breeding colony. Aquaria with all equipment must be thoroughly cleaned and disinfected to prevent spreading of diseases.

Selective breeding may produce strains with improved resistance towards some of the infectious diseases (ICES 2004). Disease incidence can furthermore be reduced by sterilising natural seawater with UV radiation. Seawater should be filtered through sieves with 1 µm mesh size prior to UV- sterilization. Appropriate UV appliances can be purchased from aquarist suppliers.

Assuming initial signs indicate health disorders, further disease propagation (potentially affecting the whole breeding colony) might be prevented by maintaining oysters at reduced salinity (< 1.5%) and temperature (ICES 2004). It has been proven effective to reduce the population density in breeding tanks and to enhance the seawater flow rate. Antibiotics and other pharmaceuticals must not be used in disease treatment of molluscs used in OECD tests as some active agents may well impact the endocrine system and therefore would render the organisms useless for ecotoxicological purposes.

Dead oysters must be frequently removed from the breeding colony. If there is a breeding aquarium with enhanced mortality, (according to Council Directive 95/70/EC sudden mortality affecting approximately 15% of stocks and occurring over a short period between two inspections (confirmed within 15 days) (EU 1995) all oysters must be excluded from the breeding colony and humanely killed by overdosing of narcotics (e.g. 24 hours exposure to 7.5% $MgCl_2$ solution).Afterwards the aquaria and all equipment must be cleaned and disinfected thoroughly. In any case, the introduction of diseased oysters into natural environments must be avoided.

### 1.27 Data gaps

This paragraph is intended to characterise gaps in knowledge identified for improved breeding and culture management with regard to the three candidate species. Some of them might be appropriately addressed with additional research activities and generation of new data, but others data gaps are simply due difficulties in gaining access to data and expert knowledge that already exist.

#### 1.27.1 Adjustment of industrial aquaculture techniques

This issue becomes apparent when evaluating different instructions for bivalve culture (e.g. FAO 2004, Henley et al. 2001, Robert and Gerard 1999, Soletchnika et al. 2000). Depending on species and aquaculture technique there are many protocols in existence in countries where aquaculture has a long tradition (especially France and Japan). However, such guidelines are not necessarily publicly available, are often written in the respective national language and are tailored to comply with standards for mass production for aquaculture industry purposes. In any case it would be a challenge to adjust these "industrial" methods to the laboratory scale (T. Horiguchi, National Institute for Environmental Studies, Ibaraki, Japan, pers. comm.), a problem made all the more difficult if local and logistical conditions require a fall back to the use of artificial seawater. Purchase of adult, sexually mature and unexposed *Crassostrea* from commercial hatcheries could mean an alternative to reduce breeding expenses but this would also mean that quality control requirements would have to be defined, applied and certified.

#### 1.27.2 Basic research

Another crucial point is the need to collate currently existing data, or to generate new experimental data, on unsettled questions concerning the basic biology of the candidate species. To differentiate between detrimental, chemically-induced effects on the candidate species' endocrine systems and natural, non-significant variations above or below a mean value requires definition of the normal standard state for endpoints under investigation. As already mentioned, these studies would have to include the characterization of an annual reproductive or developmental cycle under defined laboratory conditions, food, temperature, salinity and conductivity preferences, mate choice and mate selection (*Lymnaea stagnalis*).

#### 1.27.3 Sensitivity of genetically different strains

More than 50 years ago Warwick (1952) reported on three different morphotypes (A, B, C) identified in British *Potamopyrgus jenkinsi* populations. Strains are morphologically distinguishable, and exhibit different ecological and physiological preferences (Jacobsen and Forbes 1997; Son et al. 2008, Warwick 1952) and it was assumed that all European *Potamopyrgus* snails belong to the New Zealand species *P. antipodarum* with the existence of very few clonal lineages in Europe (Städler et al. 2005). However, what might be more important for OECD guideline development are the findings of Jensen and Forbes (2001) on differences in susceptibility of European clones to acute cadmium exposure (48 hours at 0.0, 0.25, 0.50, 1.0, 2.0, and 4.0 mg Cd/L). The results of this study indicated that clone A ($LC_{50}$ value 1.92 mg Cd/L) and clone B ($LC_{50}$ value 1.29 mg Cd/L) are less sensitive to high cadmium concentrations than clone C ($LC_{50}$ value 0.56 mg Cd/L). According to Fromme and Dybdahl (2006) clone A invasions of Europe originated from New Zealand whereas the source of clone C is Australia. Furthermore, authors observed different responsiveness of the European clones A and C to artificial trematode infections with *Microphallus* spec. originating from Australia. As these investigations demonstrate inherent differences in susceptibility of clonal variants to selected environmental factors and cadmium, a mutual agreement on a preferred clone for testing and assessment of endocrine disrupters and other chemicals would be an option if future research also indicates sensitivity differences for organic chemicals between clonal lines.

**Implementation**

### 1.28 Animal welfare

A molluscan reproduction test should provide the largest amount of information from the lowest possible number of animals. The number of replicates should be adequate to detect significant effects of potential EDCs and substances toxic to reproduction and development. Appropriate endpoints must be considered first to ensure that as much information as possible is obtained from the test. For example, as mentioned in section 0, the demand for replicates is higher if the ANOVA statistic is used whereas (non-)linear regression generally demands more concentrations. This in turn depends on whether the aim is to calculate NOEC/LOEC or ECx values. By further refinement of existing testing methods the duration and hence the number of test individuals could be reduced, e.g. if the test with *Potamopyrgus antipodarum* were to follow the Standard Operating Procedure (SOP) of Duft et al. (2007), its duration could be reduced from 8 weeks to 4. This would result in lower test costs and still produce significant results, compared to the literature (Alonso and Camargo 2009, Duft et al., 2007, Jobling et al., 2004, but see Pedersen et al. 2009).

Test and culture conditions should be optimal. Unvarying water quality enhances the well-being and guarantees the reproduction of organisms. Frequent checking of water and test media parameters, according to the specifications in a guideline are therefore recommended (see section 0). Further, animals from the breeding group should be observed regularly. Abnormal behaviour e.g. lethargy with drooped soft body and unresponsiveness to mechanical irritation may indicate a bad health status. Animals afflicted with health problems have to be removed and should not be used for testing (see section 0).

Based on the less complex structure of their nervous systems it is generally assumed that the majority of molluscs is not able to experience pain at all, or at least in a less intensive way than vertebrates. In autumn 2005 the European Commission charged the Scientific Panel on Animal Health and Welfare of EFSA (European Food Safety Authority) with the revision of Directive 86/609/EEC on the protection of animals, and the scientific elucidation of whether invertebrates can perceive pain. Following a classification system developed by this expert panel, only cuttlefish, highly evolved Malacostraca, jumping spiders, social insects and free swimming larval forms of tunicates seem to be able to experience pain whereas no scientifically convincing evidence for pain sensation in gastropods and bivalves has been found. Therefore the use of gastropods and bivalves for the assessment of endocrine disrupters meets the requirements for standardized chemical testing within the European Union as well as animal protection considerations. Following the 3-R-Principle (Reduce, Refine, Replace) defined by Russel and Burch (1959), toxicity experiments with molluscs (except cephalopods) would fulfil the ECVAM (European Centre for the Validation of Alternative Methods)-criteria of a ‘replacement test’ (method which permits a given purpose to be achieved without using living vertebrate animals) and a ’complementary test’ (test that provides information that adds to or helps interpret the results of other tests).

Nevertheless, whenever dissection of gastropods is essential, individuals should first be narcotised in a 2.5% solution of magnesium chloride hexahydrate ($MgCl_2$ x 6 $H_2O$, comp. section 7.3.1) in deionised or distilled water, according to Duft et al. (2007).

### 1.29 Facilities and equipment

The test conditions of the experiments should be comparable to those under which the breeding stock is kept. For every test species, basic parameters like pH, oxygen concentration, temperature, conductivity and nitrite concentration of all breeding and test waters have to be measured regularly. Therefore adequate measuring instruments are needed. Further, laboratories should have enough space for both the breeding stock and performance of the test. Those with convenient facilities and the basic equipment for life-cycle tests with aquatic invertebrates may not have problems with conducting the experiments. Flow-through systems may be needed to guarantee constant availability of test substances during the testing period. For the evaluation of effects, a stereomicroscope with an ocular micrometer and dissecting dish and dissecting instruments are needed. Laboratory staff should be trained to evaluate endpoints and to have perfect command of the methods.

Table 10-1 provides a list of the essential equipment needed for the three candidate test organisms for either partial (*Potamopyrgus antipodarum*) or full life cycle testing methods (*Crassostrea gigas, Lymnaea stagnalis*). The PLC test design with *L. stagnalis* proposed by colleagues from INRA (Rennes, France) is not considered in this table (cf. Appendix 2 of this document for details, including essential equipment).

Table 10-1: Essential equipment needed for test performance with the three candidate species.

| ***Potamopyrgus antipodarum*** | ***Lymnaea stagnalis*** | ***Crassostrea gigas*** |
|---|---|---|
| 1000 mL glass beakers | 1000 mL glass beakers (for breeding of the F1 and F2 generation)<br>6-well plates, 10 mL per well, for exposure of egg clutches (one clutch per well) | 2000 mL glass beakers (for breeding and exposure of the F1 generation) |
| 10 L glass aquaria for cultivation | 10 L glass aquaria for exposure and cultivation of the F0 and F1 generation | 250 L glass aquaria for exposure of the F1 generation until adulthood<br>30-50 mL glass beakers (for F2 cross-breed experiments) |
| glass pipettes | glass pipettes | glass pipettes |
| dissolved oxygen meter | dissolved oxygen meter | dissolved oxygen meter |
| pH meter | pH meter | pH meter |
| conductivity meter | conductivity meter | refractometer |
| thermometer | thermometer | thermometer |
| stereomicroscope | stereomicroscope | stereomicroscope |
| climate chambers or temperature regulated room | climate chambers or temperature regulated room | climate chambers or temperature regulated room |

| | | |
|---|---|---|
| with temperature indicator | with temperature indicator | with temperature indicator |
| dissecting dish and dissecting instruments | | algal culturing facilities |

The partial life cycle testing method with *P. antipodarum* requires less space due to the need for a smaller number of containers than full life cycle tests with *L. stagnalis,* and *C. gigas* in particular. It is also faster to conduct (28- or 56-day exposure versus 217 and 280 days). Both considerations also apply to the proposed PLC testing method with *L. stagnalis* in Appendix 2. Additionally, feeding of *P. antipodarum* and *L. stagnalis* with TetraPhyll® and Tetramin® is less susceptible to problems than feeding of *C. gigas* which requires the cultivation of algal cultures.

### 1.30 Running costs

Running costs are based on the salaries of staff who take care of the breeding stock, conduct and evaluate the test, water consumption, food costs and maintenance charges in general, including facilities and energy use. They may vary depending on the organism and testing method chosen, and on the particular testing laboratory, so absolute costs cannot be given. Higher food costs are to be expected for *Crassostrea gigas* because healthy algal stocks have to be maintained. Running costs also increase with the time of test exposure. Again, in comparison with the other organisms mentioned in Table 10-1, the long test duration for *C. gigas* leads to the highest costs.

Compared with each other, the *Potamopyrgus* partial life cycle test would incur low running costs (comparable to the the proposed PLC testing method with *L. stagnalis* in Appendix 2), the *Lymnaea* full life cycle test medium costs, and the *Crassostrea* full life cycle test high running costs. Further information on costs is presented in section 1.33.

### 1.31 Test reproducibility and confounding factors

For *Potamopyrgus antipodarum*, several studies suggest that it gives consistent results: e.g. in a triphenyltin contaminated sediment a decline of the number of unshelled embryos in a time- and concentration-dependent manner was shown (Duft et al., 2003a), and similar results were recorded when animals were exposed via water in the EU-project COMPRENDO (project code: EVK1-CT-202-00129). Again for *P. antipodarum*, an increase in embryo production was also induced by octylphenol in water (Casey 2000) as well as via sediment exposure (Duft et al., 2003b). This clearly shows an impact of these test substances on the same endpoint, namely embryo production, even when test designs vary. Besides, other studies reveal that *P. antipodarum* is a test organism that is easy to handle (Jobling et al., 2004; Mazurova et al., 2008).

However, despite this experience with *P. antipodarum*, none of the three proposed test procedures have yet been subjected to formal intercalibration trials aimed at measuring reproducibility in a rigorous manner.

The reproducibility of experimental data obtained from molluscs depends on a variety of factors, as follows:

a) Animal choice: It is essential that individuals collected from unpolluted field sites undergo an acclimatisation phase before being employed in a test. Ideally and to avoid seasonal variations in reproduction (see below), their acclimated offspring reared under controlled laboratory conditions should be used for testing.

b) Animal behaviour: Juvenile and adult *Lymnaea stagnalis* exhibit cannibalism which may result in

experimental bias (e.g. by increased growth and/or fecundity of cannibal snails).

c) Seasonal variations: Variations within seasonal cycles of different endpoints (e.g. egg/embryo production) may even occur after acclimatisation periods of months in the laboratory. Therefore, before starting a test the reproductive output should be determined to identify the normal state. This may help to decide whether effects of test chemicals are masked by suboptimal choice of exposure periods (Duft et al., 2007): If a *Potamopyrgus antipodarum* test is conducted during a phase with decreasing embryo numbers in control animals, the effects of reproductive toxicants may be less visible, while the stimulating effect of estrogenic chemicals on embryo production may be partially or totally masked during phases of increasing embryo numbers in control animals.

d) Parasitic infections: As mentioned in section 0, many molluscs are susceptible to parasitic infections, some of which can cause conditions that mimic endocrine disruption, or otherwise influence the molluscan endocrine system. Efforts should therefore be made to eliminate parasites from culture systems.

e) Food quality: Together with other stress factors, reduced food quality and quantity can lead to small or weakened progeny. Culturing of *Crassostrea gigas* or *Ostrea edulis* requires abundant supplies of clean seawater and the ability to grow several species of marine micro-algae as a food supply. An inadequate algal stock can therefore compromise a whole experiment.

f) Reliability of test variables: Appropriate training of staff, transparency of data and employment of proper statistics are basic requirements contributing credibility to the test outcome.

The results obtained from an experiment may vary depending on one or more of these factors; consequently they may have an influence on data interpretation. Study directors should therefore be familiar with the testing procedure and evaluate possible interferences before interpreting data.

### 1.32 Data interpretation

There are several aspects which should be considered regarding data interpretation when using gastropod and bivalve species in biotests that may be developed for OECD.

#### 1.32.1 Representativeness and data extrapolation

Do molluscs provide appropriate models to allow for risk and hazard assessment beyond their phylum and for conclusions to be made about ecosystem health?

Molluscs represent one of the most diverse and species-rich phyla of the animal kingdom. Due to their ubiquitous distribution and high species number, molluscs play important ecological roles in the different aquatic and terrestrial ecosystems of the world. They include key species for ecosystem functioning (e.g. litter decomposition), and constitute huge amounts of the biomass at different trophic levels in ecosystems (from primary consumers to top predators).

In contrast to their ecological importance, and despite numerous reports about the occurrence of endocrine disruption in molluscs, the phylum is under-represented in studies of chemical toxicity in the laboratory. This is especially true for the routine hazard testing of chemicals, where species from other invertebrate groups like arthropods, mainly crustaceans, are much more widely used. However, comparative studies of vertebrates and invertebrates (including molluscs) exposed to EDCs and other environmental chemicals suggest that reproductive and developmental effects of test compounds are often similar. Although species-specific differences in effect patterns and sensitivity of test species do occur, experiments have revealed that many test substances have similar impacts on apparently endocrine-related endpoints in both vertebrates and invertebrates (comp. Albanis et al., 2006; Crain et al., 2007 ; Jobling et. al., 2004 ; Lagadic and Caquet, 1998). In various studies, molluscs have turned out to be remarkably susceptible to endocrine

disrupters and to other environmental chemicals. This may partly result from the fact that molluscs are effective bioaccumulators of chemicals, and are provided with relatively ineffective detoxification enzymes (e.g. MFO system) which result in their increased sensitivity compared with several other systematic groups (Oehlmann and Schulte-Oehlmann, 2003b).

### 1.32.2 Environmental relevance

To what extent is it possible to draw valid conclusions from laboratory tests with the candidate species about effects in the field?

For effects caused by endocrine disruption, the relevance of conclusions from laboratory tests for field situations may be high. Substances interfering with the endocrine system at any level, and perhaps especially those which damage the control of reproduction, have the potential to cause negative effects on populations and their risk assessment should therefore be taken seriously.

The best example of such linkage between laboratory and field concerns the organotins (see sections 0 and 0), which not only cause masculinisation of some female molluscs in the laboratory, but which have eliminated or reduced many wild mollusc populations, effects which have also resulted in community-level damage.

Nevertheless, it is also true that few other studies have been made which firmly link endocrine disruption in molluscs with adverse effects on ecosystems. As with many other types of toxicity and many other species, there is a general need in ecotoxicology for more studies which examine these links, in order to place environmental risk assessment on a firmer basis. Many molluscs are easily studied in mesocosms, so the tools are available to fill this gap in knowledge.

### 1.32.3 Hormesis or low dose effects

How to treat data suggesting a positive effect on species performance at low doses, and how to distinguish this from hormesis?

Among toxicologists and ecotoxicologists, a fundamental concept in risk assessment and regulation is the dose-response or concentration-response relationship. The threshold model has traditionally been used to assess risks of non-carcinogens while the linear non-threshold model was applied on low doses of carcinogens (Calabrese and Baldwin, 2003a). The U-shaped model, commonly called hormesis, has not often been considered.

In general, a hormetic response is a stimulatory response of an organism exposed to low concentrations of a toxicant while an inhibition occurs at much higher concentrations (biphasic model). Many estrogenic responses have a dose-response relationship that could be described as hormetic (Weltje et al., 2005). Bisphenol A is an example of a xeno-estrogen which causes unique effects at low but not at higher concentrations *in vivo* in the freshwater snail *Marisa cornuarietis* (Oehlmann et al., 2000, but see Forbes et al 2008a) as well as in adult mice (Judy et al., 1999) and *in vitro* in human prostatic adenocarcinoma cells (Wetherill et al., 2002).

Hormetic responses and low-dose effects of EDCs both have U-shaped dose-response curves though endocrine disruptors are defined by their mechanism, namely a type of interference with some part of the endocrine system of an organism. Such disruption is marked by an increase or decrease of the response compared to the control group. By comparison, hormetic responses may modestly overshoot the original homeostatic set point, resulting in a low-dose stimulatory response (Calabrese 1999), which is regarded as an adaptive or even beneficial reaction in order to stimulate protective mechanisms in general, independently of the chemical exposed.

Weltje et al. (2005) conclude that the commonly used threshold dose-response model underestimates risk

through estimating low-dose responses from high-dose studies (also see Welshons et al., 2003). A difficulty is that the magnitude of the low-dose stimulation in the hormetic model is usually quite small and therefore hard to measure, and is often regarded as normal variability (Calabrese and Baldwin, 2003b). In designing studies (including mollusc reproduction tests) for risk assessment of suspected EDCs, a wide range of lower concentrations in particular may therefore need to be examined (vom Saal and Hughes, 2005) and more replicates may be required to optimise statistical power (Calabrese and Baldwin, 2003a).

Furthermore, an affected organism responds to damage signals with a coordinated series of temporally mediated repair processes (Calabrese, 2008). A possible hormetic response can be optimally evaluated by assessing the dose-response process over time. Experiments that include only one time point for evaluation are as inapplicable as testing with too few or too high doses or inadequate dose spacing (comp. section 0 for dose selection). Repeated measures of appropriate endpoints are therefore recommended.

Hormesis/ low-dose effects are an issue which does not solely apply to EDCs or molluscs, but it is relevant for consideration here because it has been observed in some mollusc reproduction experiments as mentioned above. Implementing the hormesis concept in risk assessment is a challenging task as it implies that exposure to low doses of toxic chemicals could be good for an organism, a view that in the past has led to the suggestion that risk assessment is overprotective and causes unnecessary fear of exposure to traces of chemicals (Weltje et al., 2005).

### 1.33 Advantages and disadvantages of the different candidate species

All three candidate species are known to be robust and suitable for laboratory testing, although the reproducibility of the tests remains to be fully established. Their advantages and disadvantages are summarized in Table 10-2:

Table 10-2: Summary of advantages and disadvantages of the three candidate test species.

| | **Advantages** | **Disadvantages** |
|---|---|---|
| *Potamopyrgus antipodarum* | Sexual maturity is reached within 3 months under optimal conditions | Biomass is insufficient for possible biomarker studies |
| | Breeding stock is easy to handle and does not need special equipment | Fecundity is the only apical endpoint for partial life cycle testing |
| | High sensitivity to a substantial range of EDCs | Delicate handling required during opening of brood pouch |
| | Long cleaning intervals (7-14 days) | |
| | High population density (150 individuals/L) | |
| | Breeding stock requires little space (5 $m^2$) | |

| | Advantages | Disadvantages |
|---|---|---|
| | Short test duration (4-8 weeks) | |
| | SOP already available | |
| | Low culturing costs | |
| *Lymnaea stagnalis* | Sexual maturity is reached within 3 to 4 months | Full life cycle test duration of 31 weeks |
| | Breeding stock is easy to handle and does not need special equipment | Tank size for culturing is up to 100 L and needs a lot of space |
| | Large variety of endpoints | Low population density (5 individuals/L) |
| | Biomass sufficient for biomarker studies. | Need to choose between many possible endpoints |
| | Known sensitivity to a substantial range of EDCs | |
| *Crassostrea gigas* | Long cleaning intervals (7- 14 days) | Ideally cultivation requires access to abundant supplies of natural,clean seawater |
| | Large variety of endpoints | Algal culturing facilities are needed to provide a food source |
| | The only bivalve test and the only marine species currently suitable for guideline development, | Large tank size (up to 500 L) required for culture of the breeding stock, as well as substantial space (15 $m^2$) |
| | Species of commercial relevance | Low population density (0.2 individuals/L) |
| | | Long time until sexual maturity is attained (7-18 months) |
| | | Long test duration (16-28 weeks) |
| | | Data on sensitivity to only a small range of EDCs |

According to the points itemised in the table above, the *Crassostrea gigas* test seems to be the most complex and the least promising. On the other hand, *C. gigas* is of more relevance for humans as the species serves as a food source. It is also the only marine candidate species, and the only bivalve.

Using *Lymnaea stagnalis* may be disadvantageous: When performing single exposure tests, snails have to be used which have been cultivated separately, otherwise it remains unknown if the progeny are a product of self-fertilization or of sexual reproduction. However, a brood with individually kept animals is very costly, space- and time-consuming. When comparing *Potamopyrgus antipodarum* with *L. stagnalis*, the mudsnail shows a higher sensitivity to EDCs than *L. stagnalis* (e. g. LOEC for EE2 is 1 ng/L in *P. antipodarum* compared with the LOEC of 500 ng/L for the same compound in *L. stagnalis*; comp. section 5.2).

Regarding costs, the *P. antipodarum* partial life cycle test is expected to be the cheapest with an eight weeks test duration in a semistatic system. Testing *L. stagnalis* is much more time-consuming because clutches have to be collected daily (weekends included). Compared with *P. antipodarum*, the brood stock needs more space due to the animals´size. Both *P. antipodarum* and *L. stagnalis* are easier and cheaper to breed and to test than *C. gigas*. The oyster needs special algal cultures as a food source and ideally natural sea water as culture medium, two requirements which, together with the relatively long test duration for *C. gigas*, enhance the cost and complexity of using this species.

If presented in terms of the time required to run each test, the current *P. antipodarum* protocol takes about 72 h of technician time and 8 h for data evaluation (statistics; calculation of effect concentrations; reporting); the *L. stagnalis* test takes about 175 h technician time and 8 h data evaluation. There is much less experience with running *C. gigas* life cycle tests, but it is estimated that they will take at least 250 h technician time and 8 h data evaluation. Culturing times for *P. antipodarum* and *L. stagnalis* are not included in the above estimates, and are anticipated to take about 5 h per week. Culturing in-house is not necessary for *C. gigas*, as adult oysters from known genetic lines can be obtained routinely from specialist hatcheries.

## Conclusions

### Need for mollusc-based tests of EDCs and other chemicals

I. New legislation on the registration of chemicals is now in force, or shortly will be, in several jurisdictions including the European Union, the United States and Japan. One common and novel feature is a desire to detect EDCs, to assess their environmental hazards, and to predict the likely environmental risks which they pose. However, as yet there are few standardised ecotoxicity tests available with adequate sensitivity to EDCs, although 3 relevant Guidelines have recently been published by OECD (OECD, 2009a, b and c). Standardised invertebrate tests with sensitivity to EDCs are also still in development, but these currently involve just a single phylum, the arthropods. Because of the requirement to protect all organisms from the harmful effects of chemicals, there is a need for a wider range of such tests, covering other important invertebrate phyla. It is **concluded** that the molluscs probably offer the best opportunity at present for developing new test methods that are sensitive to both EDCs and non-EDCs. The term 'EDC' here applies not only to substances with possibly unique endocrine action in molluscs, but also to some substances that are known to disrupt the endocrine systems of vertebrates. Furthermore, given that there are more than 130,000 species of mollusc which are distributed across almost all habitats, it is also **concluded** that the results from such tests should be applicable to many ecologically and economically important taxa in this phylum.

### Understanding of molluscan endocrinology

II. Like in all other multi-cellular organisms, many important metabolic processes in molluscs are under the control of an endocrine system. Furthermore, this system appears to have many features in common with those in other phyla, including the chordates, with strong evolutionary conservation of some hormones. However, detailed knowledge of the endocrine system in molluscs is fragmentary, and based on studies of a small range of species. While the primary molluscan hormones are generally a range of peptides produced by secretory centres in various neural ganglia, several groups also employ vertebrate-type steroids which have functional roles but which appear to act in different ways to the well-known mechanisms in vertebrates. Furthermore, there are wide differences in the details of hormonal control across the various molluscan groups. The result of this incomplete knowledge is that we have little insight into how EDCs act in molluscs, an observation exemplified by the case of tributyltin for which there are five competing hypotheses about its MOA. It is therefore **concluded** that it would be premature to develop mollusc-based screening tests for EDCs, as these demand an understanding of MOA in order to diagnose correctly the type of EDC in question. For similar reasons, it is also **concluded** that we cannot at present use mollusc-based tests as surrogates for fish-based procedures, although there may be scope for this in the longer term.

### Responses of molluscs to EDCs

III. There is good evidence that many aquatic molluscs are experiencing endocrine disruption in the field, or at the least, interference at low concentrations with processes such as reproduction that are

under endocrine control. This evidence is drawn primarily from the case of the organotins which have caused widespread masculinisation of female molluscs, and which has led to population declines and some local extinctions in many prosobranchs and some bivalves. There is much less field evidence for other types of endocrine disruption in molluscs, but exposure to estrogens or their mimics appears to have caused intersex and vitellin induction in some species, in a manner analogous to similar effects seen in fish. These field observations have been backed up by much laboratory-based experimentation, and additional studies suggest that substances able to interact with vertebrate androgen receptors can also cause adverse effects in some molluscs. Finally, there are limited experimental data suggesting that some polycyclic aromatic hydrocarbons (PAH) can delay gametogenesis in molluscs by interfering with steroid metabolism. Crucially, many laboratory-based experiments have shown that organotins and estrogens can be very potent in molluscs. It is therefore **concluded** that some molluscs have sufficient sensitivity to certain EDCs to justify the international standardisation of test methods based on this phylum. However, given the poor understanding of MOAs, it is also **concluded** that such standardisation should be restricted at present to apical tests that are likely to detect the adverse effects of EDCs (and other chemicals) without necessarily diagnosing mechanisms.

**Available types of molluscan test procedure**

IV. The two types of apical test with molluscs with which there is the most experience are partial life cycle (PLC) tests and full life cycle (FLC) tests. By definition, PLC tests only cover part of the lifecycle, generally the phase of reproduction (e.g. F0 adults to F1 eggs or juveniles), whereas FLC tests cover all stages including growth, sexual development and reproduction. It is presumed that most endocrine disrupters will be detected by single-generation FLC tests (e.g. F0 adults to F1 adults), although highly bioaccumulative EDCs may be even more potent in multi-generation FLC tests (e.g. F0 adults to F2 juveniles or adults) in which offspring can be exposed via maternal transfer of residues as well as via the ambient water or sediment. PLC tests are necessarily cheaper than FLC tests, and although they may also be less sensitive to EDCs, it is **concluded** that the standardisation of mollusc-based tests using both PLC and FLC approaches should be investigated further.

**Test procedures recommended for further optimisation and validation**

V. Many factors need to be taken into account when choosing suitable species and procedures for standardisation, including ethical considerations, sensitivity to EDCs, cost, practicality, availability of test organisms, reproducibility, statistical power, and scope for extrapolation of the results to other species. These factors (which are not well-characterised in all cases) have considerably restricted the choice available, but it is **concluded** that five test procedures show promise for standardisation and validation, although each has disadvantages as well as advantages. The first is a PLC test with the ovoviviparous freshwater gastropod *Potamopyrgus antipodarum*. This is the mollusc-based EDC test for which the most experience exists, and a standard operating procedure is already available. Exposure to test material in this test can be either via the ambient water or via spiked sediment. The second is an FLC test with *P. antipodarum*, but there is at present much less experience with this procedure, and a little more work is required to establish an agreed protocol. The third is an FLC test with the hermaphroditic freshwater pulmonate *Lymnaea stagnalis*. There is good experience in EDC testing with this species, but the proposed operating procedure needs to be investigated more thoroughly. The fourth is a PLC test with *L. stagnalis*, but there is even less experience with this at present than with the equivalent FLC test, and again, a suitable protocol has not yet been finalised (although an as yet unpublished PLC test with this species is described in Appendix 2). The fifth is an FLC test with the marine bivalve *Crassostrea gigas*. There is much less experience of EDC testing with this species than the other two, implying the need for considerable further development work, but it offers the potential benefit of being the only marine species chosen, and the only bivalve.

**Recommended priorities for optimisation, validation and research**

VI. Possible culture and test operating procedures for the three chosen species have been set out in this review, but none of these can yet be considered ready for the drafting of Guidelines because of various data gaps. Probably in the cases of *P. antipodarum* and *L. stagnalis*, even though some tested SOPs are already available, and certainly with *C. gigas* where the proposed SOP remains more tentative, it would be beneficial to conduct further research to optimise test conditions more precisely. It is known that mollusc reproduction can be extremely sensitive to such factors as temperature, daylength, population density and food quality, and it is certain that their responses to EDCs can be significantly modulated by these factors. Given that the *P. antipodarum* PLC procedure is the best understood, it is **concluded** that this should be given priority for further test optimisation and validation, activities which could most efficiently proceed simultaneously. A particular issue for optimisation in this species concerns the influence of temperature on the sensitivity of (increased) fecundity to estrogens. Concerning validation of this method, the most pressing need is for the investigation of statistical power and inter-laboratory reproducibility with a range of EDCs, non-EDCs and negative substances. It is **concluded** in the case of the other four methods that their test conditions and relative sensitivities should be more fully defined before decisions are taken about possible validation. In particular, research should be conducted to decide, (a) if FLC tests with *P. antipodarum* and *L. stagnalis* are consistently more sensitive to EDCs and other chemicals than the respective PLC tests; and (b) to decide if some groups of chemicals are consistently more potent in *L. stagnalis* or *C. gigas* than *P. antipodarum*. Longer-term research would also be desirable to identify other possible test species (e.g. the tropical ramshorn snail *Biomphalaria glabrata*), including one or more sexually-dimorphic species whose sensitivity to EDCs may differ substantially from the species recommended in this review. Longer-term strategic research is also urgently needed to understand more fully the endocrinology of mollusc species which might ultimately support the development of diagnostic screening tests for EDCs.

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**List of abbreviations**

| | |
|---|---|
| ACP | Advisory Committee on Pesticides, UK |
| ACTH | Adrenocorticotropic hormone |
| ALP | Alkali-labile phosphate |
| ANOVA | Analysis of variance |
| APGWamide | H-Ala-Pro-Gly-Trp-$NH_2$, a tetrapeptide neurotransmitter in molluscs |
| AR | Androgen receptor |
| ASTM | American Society for Testing and Materials |
| ATAT | Acyl coenzyme A testosterone acyltransferase |
| BPA | Bisphenol A |
| CaBP | Calcium-binding protein |
| CBG | Cortisol binding globulin |
| CDCH | Caudo-dorsal peptide hormone (CDCH-1: ovulation hormone) |
| CNS | Central nervous system |
| CoA | Acyl coenzyme A |
| CPA | Cyproterone acetate |
| CRH | Corticotropin-releasing hormone |
| CV | Coefficient of variation (standard deviation / mean x 10) |
| CYP19 | Cytochrome P450- dependent aromatase |
| DBH | Dorsal body hormone |
| DMSO | Dimethyl sulfoxide |
| DOE | Department of the Environment |
| DRP | OECD Detailed Review Paper |
| E2 | 17β-estradiol |
| EDC | Endocrine disrupting chemical |
| EDTA | OECD Endocrine Disrupter Testing and Assessment Taskforce |
| EE2 | 17α-ethinylestradiol |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Estrogen receptor |

| | |
|---|---|
| EU | European Union |
| FEN | Fenarimol |
| FLC | Full life cycle |
| FMRFamide | H-Phe-Met-Arg-Phe-$NH_2$, a tetrapeptide neurotransmitter in molluscs |
| GHBP | Growth hormone binding protein |
| GnRH | Gonadotropin-releasing hormone |
| $HT_{50}$ | Mean hatching time |
| ICES | International Council for the Exploration of the Sea |
| IMO | International Maritime Organisation |
| ISO | International Organisation for Standardisation |
| JH | Juvenile hormone |
| $K_{OC}$ | Organic carbon normalized sorption coefficient for soils and sediments |
| $K_{OW}$ | Octanol/water partition coefficient |
| L-EGF | *Lymnaea* epidermal growth factor |
| LOEC | Lowest observed effect concentration |
| LTI | Lymnaea trypsin inhibitor |
| MIP | Molluscan insulin-like peptide |
| MOA | Mode or mechanism of action |
| MT | Methyltestosterone |
| NOEC | No observed effect concentration |
| NP | Nonylphenol |
| NSC | Neurosecretory cells |
| OECD | Organisation for Economic Cooperation and Development |
| OP | Octylphenol |
| PAH | Polycyclic aromatic hydrocarbon |
| PCB | Polychlorinated biphenyl |
| PCP | Pentachlorophenol |
| PLC | Partial life cycle |
| PMF | Penis morphogenic factor |
| PRF | Penis regression factor |
| proELH | Egg-laying prohormone |
| RA | 9-cis retinoic acid |
| REACH | EU regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals |

| | |
|---|---|
| RXR | Retinoid X receptor |
| SHBG | Steroid hormone binding globulin |
| SIS | Sodium influx stimulating peptide |
| SPSF | OECD Standard Project Submission Form |
| T | Testosterone |
| TBG | Thyroxine binding globulin |
| TBT | Tributyltin compounds |
| TPT | Triphenyltin compounds |
| TRH | Thyroid-stimulating releasing hormone |
| UBA | Umweltbundesamt (German Federal Environment Agency) |
| USEPA | United States Environmental Protection Agency |
| WNT | OECD Working Group of National Coordinators of the Test Guidelines Programme |

**Appendix 1: Comparison of available test methods with molluscs**

Detailed comparison of the attributes of different available test methods (partial and full life cycle) with molluscs. For convenience, this table has been split into 3 sections: a) Marine and Freshwater Bivalves; b) Marine Gastropods; and c) Freshwater Gastropods.

**a) Marine and freshwater bivalves**

| **Criterion / species** | ***Dreissena polymorpha*** | ***Scrobicularia plana*** | ***Crassostrea gigas*** | ***Ostrea edulis*** | ***Mytilus edulis*** |
|---|---|---|---|---|---|
| **Species charcterization** | | | | | |
| Freshwater/estuarine/marine | freshwater/estuarine | marine/estuarine | marine/estuarine | marine | marine |
| Robust (suitable for lab testing) | yes | yes | yes | yes | yes |
| Gonochoristic (separate sexed) | yes | yes | yes (consecutive-protandric hermaphrodite) | yes | yes |
| Both sexes occur | yes | yes | yes (sex change possible) | yes | yes |
| **Culture and lab breeding** | | | | | |
| Perpetuation of | no | no | yes | yes | no |

| **Criterion / species** | ***Dreissena polymorpha*** | ***Scrobicularia plana*** | ***Crassostrea gigas*** | ***Ostrea edulis*** | ***Mytilus edulis*** |
|---|---|---|---|---|---|
| breeding stock | | | | | |
| Time to sexual maturity | 9-12 months | 12-36 months | 7 –18 months | 24-36 months | 6-12 months |
| Generations per year | 4 | 2? | 2 | 1-2 | 2? |
| Estimated annual offspring per female/individual | - | - | X $10^6$ | x $10^6$ | - |
| Ease of handling | + | ± | + | + | ± |
| Special equipment needed | Algal culturing | Algal culturing | Algal culturing | Algal culturing | Algal culturing |
| Cost of culture* | 3 (adult) | ? | 3 | 5 | (?) |
| Temperature regime | 10-20 °C | 15±1 °C | 8-18 °C (depending on season), 20±2 °C, 24 °C | 18 °C | 10-12 °C |
| Light regime (light-dark period) | 12/12 hours | 12/12 hours | 12/12 hours | 12/12 hours | ? |
| Culture medium | Reconstituted water, dechlorinated tap water | Natural or reconstituted seawater | Spat and cockle-shell filtered seawater in a flow-through system, natural seawater | Natural seawater | Filtered sea water |
| Food/feeding | Cultured estuarine/fresh-water (*Spirulina*, | No feeding or *Isochrysis galbana* (alga) | Mixed algal supply of *Isochrysis galbana*, | Mixed algal supply | *Isochrysis galbana*, *Rhodomonas spec*. (alga) |

| **Criterion / species** | ***Dreissena polymorpha*** | ***Scrobicularia plana*** | ***Crassostrea gigas*** | ***Ostrea edulis*** | ***Mytilus edulis*** |
|---|---|---|---|---|---|
| | *Chlorella*) alga daily, *Phytoplex* bivalve food | suspension | *Pavlova lutheri*, *Chaetocerus mureli* daily | | suspension every second day |
| Cleaning intervals | 5-7 days | ? | 7-14 days | 7-14 days | ? |
| Tank size for culture | 60 L | - | 100 L | 100 L | - |
| Total space required for breeding stock | 5 $m^2$ | - | 15 $m^2$ | 15 $m^2$ | - |
| Population density | ≤ 1–40 individuals/L | ≤ 6 individuals/kg | 20/100 L | 30/100 L | ? |
| **Exposure and testing** | | | | | |
| Tank size for exposure | 15 L | 12 L | 30 L; flow-through system | 30 L | ? flow-through system |
| Total required space (exposure) | 10 $m^2$ | ? | 20 $m^2$ | 20 $m^2$ | ? |
| Possible endocrine mediated endpoints | Vitellin-like proteins, oocyte degeneration, gonad histology | Intersex | Vitellin levels, P450-aromatase activity, sperm motility, sex ratio, embryo production | Embryo production | P450-aromatase activity, esterified steroid titres, downregulation of gene transcription for estrogen receptor MeER1 isoform, Vtg-like protein (ALP) |
| Life stage exposed (e.g. egg/juvenile/adult) | adult | adult | juvenile, adult | adult | adult |

| **Criterion / species** | ***Dreissena polymorpha*** | ***Scrobicularia plana*** | ***Crassostrea gigas*** | ***Ostrea edulis*** | ***Mytilus edulis*** |
|---|---|---|---|---|---|
| Apical endpoints for partial life cycle tests | ? | Intersex incidence? | Fecundity, embryo development and survival | Fecundity | ? |
| Biomass sufficient for biomarker studies | yes | yes | yes | yes | yes |
| Exposure via water | yes | no | yes (and food) | yes (and food) | yes |
| Exposure via sediment | no | yes | no | no | no |
| Test sediments | not applicable | Sieved native surface sediments (for passive & active biological effect monitoring) | not applicable | not applicable | not applicable |
| Number of replicates | 3 | ? | 2 | 2 | ? |
| Specimens per replicate | 8 | 30 | 5; 10 | 20 | 20 |
| Test duration | 15 weeks | 6 weeks | 24-48 h (adult), 16 weeks; 28 weeks (juvenile) | 25 weeks | 3 weeks |
| Life cycle test (yes/no) | no | no | possible but expensive | no | no |
| Special equipment needed | alga culture facility | alga culture facility | alga culture facility | alga culture facility | alga culture facility |
| Protocols available | Material and methods sections in publications | Material and methods sections in publications | Material and methods sections in publications | Material and methods sections in publications | Material and methods sections in publications |

| **Criterion / species** | ***Dreissena polymorpha*** | ***Scrobicularia plana*** | ***Crassostrea gigas*** | ***Ostrea edulis*** | ***Mytilus edulis*** |
|---|---|---|---|---|---|
| Cost of a test* | 4 | ? | > 5 | > 5 | ? |
| Literature | Wright et al. (1996), Quinn et al (2004, 2006), Binelli et al. (2001, 2004), Baumann (2008 ; in German) | Ruiz et al. (1994), Chesman & Langston (2006), Langston et al. (2007) | Thain & Waldock (1985), Li et al. (1998), Le Curieux-Belfond et al. (2001), Nice et al. (2000, 2003), Nice (2005) | Thain & Waldock (1986) | Arab et al. (2004), Sunila et al. (2004), Lavado et al. (2006), Ortiz-Zarragoitia & Cajaraville (2006), Canesi et al. (2008) |

* this is a relative scale, with 1= cheap (<€1,000 or US$1,500) and 5 = expensive (>€10,000 or US$ 15,000)

b) **Marine gastropods**

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| **Species charcterization** | | | | | | | | | |
| Freshwater/ estuarine/ marine | marine | marine | marine | marine | marine/ estuarine | marine/ estuarine | marine/ estuarine | marine | marine |
| Robust (suitable for lab testing) | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Gonochoristic (separate sexed) | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| Both sexes occur | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| **Culture and lab breeding** | | | | | | | | | |
| Perpetuation of breeding stock | no | yes | no | no | difficult but possible | no | no | yes | yes |
| Time to sexual maturity | 12-24 months | 24-36 months | 24 months | 36-48 months | 36 months | 12-18 months | 3-4 months | 50 days | 36 months |
| Generations | 1 | 1 | 1 | 1 | 1 | 1 | 1-2 | 2-3 | 1 |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| per year | | | | | | | | | |
| Estimated annual offspring per female | - | x $10^4$ (adults in breeding stock); juveniles: no output | - | - | X $10^3$ | - | - | x $10^3$ | x $10^6$ |
| Ease of handling | ± (culture easy; breeding very difficult) | ± (breeding difficult) | ± (culture easy; breeding impossible) | ± (culture easy; breeding impossible) | ± (culture easy, breeding easy but decreases with length in captivity and diet dependent) | ± (culture easy; breeding impossible) | ± (culture easy; breeding impossible) | + | ± (breeding difficult) |
| Special equipment needed | - | Flow-through rearing systems, large amount of unpolluted natural sea-water, several kind of filters | - | - | No filters in tanks. Flow through systems with large amount of unpolluted natural sea water or if using a static | - | - | Algal culturing | Flow-through rearing systems (200 L/h/tank, equal to 4800 L/day/tank), large amount of unpolluted natural sea- |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | system clean 2x per week | | | | water, several filters |
| Cost of culture* | 2 | Breeding stock (3 months): USD 270;<br>Juveniles (<15 mm, 6 months): USD 180;<br>Juveniles (>15 mm, 24 months): USD 730 | 3 | 3 | unknown | 3 | 3 | 3 | >5 |
| Temperature regime | 20±1 °C (18-23 °C) | Breeding stock: 20-25 °C;<br>Juveniles (<15 mm): 25 ±1 °C;<br>Juveniles (>15 mm): 15- | 15±1 °C | 15±1 °C | 10-27 °C (adults) breeding: 20-25°C; larvae: 20-25°C; juveniles: 10-27°C | 16±1 °C | 16±1 °C | 20 °C | 12-23 °C<br>(preferably 18-20 °C) |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | 25 °C | | | | | | | |
| Light regime (light-dark period) | 12/12 hours | 12/12 hours | 16/8 or 12/12 hours | 16/8 or 12/12 hours | 14/10 or 16/8 hours | 16/8 or 12/12 hours | 16/8 or 12/12 hours | 8/16 hours (8 + dim light) | 12/12 hours |
| Culture medium | Reconstituted or artificial seawater | Natural seawater | Reconstituted seawater | Reconstituted seawater | Reconstituted seawater or natural seawater | Reconstituted seawater | Reconstituted seawater | Reconstituted or natural seawater | Natural seawater |
| Food/feeding | living mussels | Breeding stock: frozen prawns and flatfish; Juveniles (<15 mm): minced *Euphausia superba*; Juve-niles (>15 mm): frozen prawns and flatfish | living mussels | bovine heart | Omnivores (Tetra Min® fish flakes ad libitum daily and/or fish, clam, mussel or shrimp meat (< 10-15 mm two times per week) ad libitum daily | lettuce; Tetra Tabi Min® tabs two times per week *ad libitum* | TetraPhyll® daily *ad libitum* | Mixed algal diet | Brown algae (e.g. *Ecklonia cava*) |
| Cleaning intervals | 7-14 days | Breeding stock: 1 day; | 7 days | 7 days | daily (static system); 3-7 | 7 days | 7 days | 7 days | 3-7 days |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | Juveniles (<15 mm): 1 day; Juve-niles (>15 mm): 2-7 days | | | days (flow-through) | | | | |
| Tank size for culture | 57-155 L | Breeding stock: 200 L; Juve-niles (<15 mm): 10 L; Juveniles (>15 mm): 57-155 L | 120 L (adults only) | 120 L (adults only) | 10-20 L (adults); 2-10 L (juveniles) | 60-120 L (adults & juveniles) | 10 L (adults only) | 50 L | 500 L |
| Total space required for breeding stock | 3-5 $m^2$ | Breeding stock: 2 $m^2$; Juveniles (<15 mm): 1 $m^2$; Juveniles (>15 mm): 1-2 $m^2$ | Not applicable | Not applicable | 1 $m^2$ (juveniles); 2-5 $m^2$ (adults and breeding) | Not applicable | Not applicable | 5 $m^2$ | 20 $m^2$ (for 6 tanks of 500 L) |
| Population density | ≤ 5 individuals/L | Breeding stock: 0.3 individuals/ L; | ≤ 5 individuals/L | ≤ 5 individuals/L | 10 individuals/L | ≤ 5 individuals/L | ≤ 150 individuals/L | 1 individual/L | 2-3 kg/500 L |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | Juveniles (<15 mm): x $10^3$- x $10^2$ individuals/L; Juveniles (>15 mm): x 10- x 1 individuals/L | | | | | | | |
| **Exposure and testing** | | | | | | | | | |
| Tank size for exposure | 2 L | 57-155 L | 60-120 L | 10-60 L | 0.25-10 L | 60-120 L | 1-10 L | 5-20 L | 155 L |
| Total required space (exposure) | 10 m² | 10-25 m² | 25 m² | 15 m² | 5-15 m² | 25 m² | 5-15 m² | 20 $m^2$ | 25 m² |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| Possible endocrine mediated endpoints | Imposex | Imposex, number/weight of egg capsules | Imposex, size of sexual glands | Imposex, size of sexual glands | Imposex, size of sexual glands and accessory organs, number of egg capsules per female, percentage of normally developing embryos, survival of veligers/ juveniles/adults, diverse biomarkers (e.g. enzyme & neurohormone expression) | Intersex, size of sexual glands | Imposex, size of sexual glands | Embryo production | Ovarian spermatogenesis, number of eggs released into seawater (fertilization rate, percentage of normally developing embryo, maybe hatching and survival rates of veliger) |
| Apical endpoints for partial life cycle tests | Imposex | Imposex | Imposex | Imposex | Imposex, fecundity, embryo development, | Intersex | Imposex | Fecundity | Fecundity, embryo development and survival |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | survival, behavior | | | | |
| Life stage exposed (e.g. egg/juvenile/ adult) | Adult, juvenile/young (for imposex induction), veliger larvae (only for acute toxicity test) | Adult, juvenile/young (for imposex induction) | adult & juvenile; passive monitoring | adult; passive monitoring | adult: (histo-logical changes, reproduction, acute and chronic toxicity tests, biomar-kers) juvenile (pathology, acute and chronic toxi-city); veliger (acute & chronic toxicity tests, settlement assays), embryo (development) | lab studies with juveniles (2-4 mm shell height only, passive monitoring, | adult; passive monitoring | Whole life cycle if required | Adult (for histological change of gonad and reproduction test), juvenile/young (for histological change of gonad), veliger larvae (for acute, subacute, chronic toxicity tests (until settlement)) |
| Biomass | yes | yes | yes | yes | yes | yes | no | yes | yes |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| sufficient for biomarker studies | | | | | | | | | |
| Exposure via water | yes | yes | yes | yes | yes | yes | yes | yes and via algal food | yes |
| Exposure via sediment | no | yes | no | yes | yes | no | Unknown but should be possible | no | no |
| Test sediments | Not applicable | to be considered | Not applicable | artificial sediments consisting of 90% quartz sand and 10% peat untreated. | Muddy sands: (1 mM mesh sieved) | Not applicable | ? | Not applicable | Not applicable |
| Number of replicates | 2-3 | 2-3 | 2-3 | 2-3 | 3-5 replicates per assay, 2 assays | ? | ? | 2 | 2 |
| Specimens per replicate | 20-50 | 20-30 | 30 | 30 | 10-15 per sex (20-30 total)? | ? | ? | 10 | 15-25 |
| Test duration (weeks) | 4-12 | 8-12 | 4-12 | 4-12 | 2-6 weeks; 1-10 days | 12-24 | 8-12 | Up to 10 weeks | 8-12 |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | (behavior ) | | | (depending on nature of test) | |
| Life cycle test (yes/no) | no | +/- (very difficult but possible, requires specifically trained staff | no | no | +/- (difficult but possible) | no | no | yes (ca. 10 weeks or 74 days) | +/- (very difficult but possible, requires specifically trained staff |
| Special equipment needed | Flow-through system, large amount of artificial seawater | Flow-through systems, large amount of unpolluted natural/artificial seawater, several filters if natural seawater is used | tide simulation, large amount of artificial seawater | large amount of artificial seawater | large amount of artificial seawater or flow through system with large amount of unpolluted, natural or artificial sea water | tide simulation, large amount of artificial seawater | - | Alga culture facility | Flow-through systems, plenty amount of unpolluted natural or artificial seawater, several kinds of filters (50 µm, 10 µm, etc.) when natural seawater is used |
| Protocols | Would have to | Would have to | Material and | Material and | Material and | ICES | Material and | Material and | Would have to |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| available | be written-up in English by Japanese colleagues | be written-up in English by Japanese colleagues | methods sections in publications | methods sections in publications | methods sections in publications | Guideline in Techniques in Marine Environmental Sciences, No. 37 | methods sections in publications | methods sections in publications | be written-up in English by Japanese colleagues |
| Cost of a test* | 2-3 | 4 | 4 | 3-4 | unknown | 5 | 4 | 2-5 (depending on nature of test) | 5 |
| Literature | Horiguchi 1993; Horiguchi et al. 1994, 1995, 1997, 1998a & b; Horiguchi et al. (unpublished) | Horiguchi et al. 2006; Horiguchi et al. (un-published) Note: Most documents on seed production & culture of juvenile ivory shells (< 15 mm) are written in | Hughes 1972, Gibbs et al. 1987, Oehlmann et al. 1996 | Stroben et al. 1992, Schulte-Oehlmann et al. 2000, Barroso et al. 2005a | Jenner 1979, Conrad 1985, Oberdörster et al. 1998, Curtis et al. 2000, Oberdörster & McClellan-Green 2000, McElroy et al. 2000, Downs et al. 2001, Gooding & LeBlanc 2001, | Williams 1964, Bauer et al. 1995, Oehlmann 2004 | Blick & Zettler 1994, Schulte-Oehlmann et al. 1997, 1998 | Calabrese & Rhodes 1974, Thain 1984 | Singhagraiwan & Doi (1993), Horiguchi et al. 1998b, 2000, 2002, 2005; Horiguchi et al. (unpublished). Note: Most documents on seed production & |

| **Criterion / species** | ***Thais clavigera*** | ***Babylonia japonica*** | ***Nucella lapillus*** | ***Nassarius reticulatus*** | ***Ilyanassa obsoleta*** | ***Littorina littorea*** | ***Hydrobia ulvae*** | ***Crepidula fornicata*** | ***Haliotis species*** |
|---|---|---|---|---|---|---|---|---|---|
| | | Japanese. These documents are useful for mass production of seeds of ivory shells at hatchery. It is unclear if techniques can be transferred to much smaller lab scale productions | | | Wayne 2001, Dickinson & Croll 2003, Straw & Rittschof 2004, Oberdörster et al. 2005, McClellan-Green et al. 2006, Sternberg & LeBlanc 2006, Curtis 2008, Finnegan et al. 2009, Sternberg et al. 2008, | | | | culture of *Haliotis* are written in Japanese (Singhagraiwan & Doi 1993 is in English). These documents are useful for mass production of abalone seeds at hatchery. It is unclear if techniques can be transferred to much smaller lab scale productions |

* this is a relative scale, with 1= cheap (<€1,000 or US$1,500) and 5 = expensive (>€10,000 or US$ 15,000)

**c) Freshwater gastropods**

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| **Species charcterization** | | | | | | | |
| Freshwater/ estuarine/marine | freshwater/ estuarine | freshwater/ estuarine | freshwater | freshwater | freshwater | freshwater | freshwater |
| Robust (suitable for lab testing) | Very difficult | yes | yes | yes | Very difficult | yes | yes |
| Gonochoristic (separate sexed) | yes | Yes | yes | yes | yes | no | no |
| Both sexes occur | yes | yes, but in Europe only females, reproduces parthenogeneticall y | yes | yes | yes | hermaphrodite | hermaphrodite |
| **Culture and lab breeding** | | | | | | | |
| Perpetuation of breeding stock | very difficult | yes | yes | difficult | very difficult | yes | yes |
| Time to sexual maturity | 4 months | 3 months | 5-8 months (depending on temperature) | 9 months | 9 months | 3-4 months | 9 months |
| Generations per | 1-2 | 3 – 4 | 3 | 1-2 | 1 | 3 | 2-3 |

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| year | | | | | | | |
| Estimated annual offspring per female | x $10^2$ | x $10^2$ | x $10^2$ | x $10^2$ | cannot be cultured at present | x $10^2$ | ? |
| Ease of handling | ± | + | + | ±/+ | cannot be cultured at present | + | + |
| Special equipment needed | - | - | - | Algal growth kit | cannot be cultured at present | - | - |
| Cost of culture* | 2 | 1 | 3 | 2 | cannot be cultured at present | 2 | ? |
| Temperature regime | 16±1 °C | 16±1 °C | 20±1 °C; 24±1 °C | 16±1 °C/ 15-20 °C | cannot be cultured at present | 20±2 °C | 15-20°C |
| Light regime (light-dark period) | 16/8 hours | 16/8 hours | 16/8 or 12/12 hours | 16/8 hours | cannot be cultured at present | 16/8 hours | ? |
| Culture medium | Reconstituted | Reconstituted | Reconstituted | Reconstituted | cannot be | 1-day old | Artificial pond |

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| | water | water | water | water or artificial pond water | cultured at present | dechlorinated tap water or M4 | water |
| Food/feeding | Cultured sessile red & green alga species | TetraPhyll® daily *ad libitum* | lettuce; TetraPhyll®, TetraMin® daily *ad libitum* | TetraPhyll® daily *ad libitum,* algal monoculture, carrots | cannot be cultured at present | TetraPhyll® , Tetramin ™ and fresh lettuce alternately twice a week | Any fish flake, up to 0.25 g/snail/day |
| Cleaning intervals | 7 days | 7-14 days | 7 days | 7 days | cannot be cultured at present | a third of the water volume is renewed every 7 | 2-3 days |
| Tank size for culture | 60 L | 10 L | 60 L | 2L, 60 L | cannot be cultured at present | 20-100 L | 10 L |
| Total space required for breeding stock | 10 $m^2$ | 5 m² | 25 $m^2$ | 10-20 $m^2$ | cannot be cultured at present | 10 $m^2$ | ? |
| Population density | ≤ 10 individuals/L | ≤ 150 individuals/L | ≤ 5 individuals/L | ≤ 10/20 individuals/L | cannot be cultured at present | ≤ 5 individuals/L | 1 specimen/L |
| **Exposure and testing** | | | | | | | |

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| Tank size for exposure | 60 L | 1 L | 60-120 L; petri dishes for embryo toxicity test | 100 mL, 2L, 60 L | ? | 10 L | 10 L |
| Total required space (exposure) | 25 m² | 2 m² | 25 m²; 1m$^2$ (embryo toxicity test) | 5-15 m² | ? | 10 m$^2$ | 10-20 m² |
| Possible endocrine mediated endpoints | Sexual maturity (pubertas praecox in males) | Embryo production | Imposex, super-females, size of sexual glands, egg production, hatching success & embryo development (em-bryo toxicity test) | Embryo development, size of sexual glands, embryo production | Embryo production | Embryo production, hatching rate, inbreeding depression, vitellogenin-like protein, histological aberration of genital organs | Embryo production |
| Life stage exposed (e.g. egg/juvenile/ adult) | adult | adult | egg, juvenile, adult | Adult | adult | egg, juvenile, adult | adult |
| Apical endpoints for partial life cycle tests | Fecundity | Fecundity | Imposex, fecundity and embryo development | Fecundity & embryo development | Fecundity | Fecundity and embryo development, | Fecundity |
| Biomass sufficient | difficult | no | yes | difficult | yes | yes | yes |

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| for biomarker studies | | | | | | | |
| Exposure via water | yes | yes | yes | Yes | yes | yes | yes |
| Exposure via sediment | no | yes | no | No | no | unknown but possible | no |
| Test sediments | not applicable | Native sediments (for biological effect monitoring) or artificial sediments: 97.5% quartz sand (mean grain size 50-200 µm) + 2.5% powdered fallen leaves of *Fagus sylvatica* or *Alnus glutinosa* | not applicable | not applicable | not applicable | Artificial and native sediments | not applicable |
| Number of replicates | ? | 4 | 2 | ? | ? | 5 | 4-10 |
| Specimens per replicate | 30 | 10-30 (depending on test duration) | 30 | 20+ | 20 | 4 | 6-9 |

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| Test duration | 24 weeks | 4-8 weeks | 12-24 weeks (adults); 8-20 days (embryo toxicity test) | 8-24 weeks | ? | 31 weeks for full life cycle test; 4 weeks for partial life cycle test proposed in Appendix 2 | 8 weeks |
| Life cycle test (yes/no) | no | no | yes | no | no | yes | unknown |
| Special equipment needed | Macro-alga culture | - | - | Algal growth kit | - | - | - |
| Protocols available | Material and methods sections PhD thesis (in German) | SOP (in OECD guideline format) | Material and methods sections in publications | Material and methods in PhD thesis ( in German) and publication | no | Material and methods sections in publications | no |
| Cost of a test* | 5 | 2-3 | 4 | 4 | - | 3-4 | ? |
| Literature | Fretter & Graham (1962), Schulte-Oehlmann (1997) | Duft et al. (2003 a, b; 2007), Mazurová et al. (2008) | Meier-Brook & Tjhen (1977), Schulte-Oehlmann et al. (1995), Oehlmann et al. (2006), Schirling | Lilly (1953), Belfroid & Leonards (1996), Schulte-Oehlmann (1997) | Susan Jobling (pers. communication) | Brown (1979), Czech et al. (2001), Segner et al. (2003) Weltje et al. (2003), Lagadic et al. (2005), Coutellec & | Ed-Routledge (pers. communication), Clarke et al. (2009) |

| **Criterion / species** | ***Theodoxus fluviatilis*** | ***Potamopyrgus antipodarum*** | ***Marisa cornuarietis*** | ***Bithynia tentaculata*** | ***Viviparus viviparus*** | ***Lymnaea stagnalis*** | ***Planorbarius corneus*** |
|---|---|---|---|---|---|---|---|
| | | | et al. (2006) | | | Lagadic (2006), Leung et al. (2007), Ter Maat et al. (2007), Bandow & Weltje (2008), De Schamphelaere et al. (2008), Ducrot et al. (2008 a, b) | |

* this is a relative scale, with 1= cheap (<€1,000 or US$1,500) and 5 = expensive (>€10,000 or US$ 15,000)

## Appendix 2: Candidate PLC test method with *Lymnaea stagnalis*[1]

### The test organism *Lymnaea stagnalis*

*Lymnaea stagnalis* (Linnaeus 1758) belongs to the clade Mollusca, class Gastropoda, sub-class Euthyneura, order Basommatophora, family Lymnaeidae. The species is common in Europe and can be found throughout northern Asia and North America (Pfleger and Chatfield, 1983). It inhabits stagnant or slow-running waters and feeds on organic constituents of plant and animal origin and is known to be cannibalistic on occasion.

The average shell height of adult snails varies according to the environmental conditions (e.g. the volume of surrounding water) but generally is in the range of 20-60 mm. *L. stagnalis* (the great pond snail) is a simultaneous hermaphrodite but the male reproductive organs mature prior to the female ones (protandric hermaphrodite). During mating, an individual can only take the male or the female part. Like all basommatophorans, *L. stagnalis* can self-fertilize. Moreover, this species shows no or very weak inbreeding depression (Puurtinen *et al.*, 2001; Coutellec and Lagadic, 2006).

Egg-masses are deposited on aquatic plants or in laboratory breeding stocks affixed to the glass surface of the aquaria. The number of eggs per clutch varies from 50 to 120 (Nichols *et al.*, 1971). Renewal of the surrounding water is known to be a trigger for egg laying (Ter Maat *et al.*, 1983).

### Principle of the test

The proposed protocol refers to a 28-day exposure test, via water, of so-called "young adults" (i.e. snails that reached maturity and began to reproduce during the test). The candidate protocol has been composed by merging experience at INRA (Rennes, France) and information from previous OECD toxicity test-guidelines. It allows for the assessment of chronic effects on reproduction (fertility and fecundity) and survival (mortality). As in *P. antipodarum* (cf. section 7.3.1), the test focuses on reproduction as an integrative endpoint. The test addresses the assessment of hormonally active substances but is not exclusively sensitive to EDCs and is equally suitable for the detection of adverse effects on reproduction mediated via other modes of action.

A brief overview of the candidate protocol is presented below:

Young adults of *L. stagnalis* are exposed to a concentration range of the test substance. The ambient water is dosed with the test substance before snails are introduced into the test beakers. Survival and reproduction (number of clutches and number of eggs per clutch) are monitored daily (or at least every other day) from the beginning to the end of the test, for 30 snails per treatment group (e.g. 6 replicates of 5 snails). Percent survival is determined at the end of the test. Mean time to first reproduction and mean cumulative fecundity per individual (i.e. number of eggs produced per individual) are also calculated, based on daily observations of reproduction. While determining the number of eggs per clutch, eggs should be carefully observed in order to assess a possible fertilization failure. Hatching rate and embryonic

[1] Appendix 2 is authored by Laurent Lagadic, Thierry Caquet, Marie-Agnès Coutellec and Virginie Ducrot (INRA, Ecotoxicologie et Qualité des Milieux Aquatiques, Rennes, France) who have developed and proposed this candidate protocol for a partial life cycle test as an alternative to the one described for *Potamopyrgus antipodarum*. The method has not yet been published or peer-reviewed.

development duration ($HT_{50}$) can be assessed as additional endpoints.

**Material and Methods**

Equipment

Test vessels and other apparatus used should ideally be made of glass or other materials chemically inert to the test chemical. All glassware must be washed thoroughly to remove any contaminants. Before washing, glassware should be sterilised by autoclaving. An overnight soak of all test equipment in DECON™ 90 (5% DECON 90 in tap water) or similar decontaminant is required, the final rinse being in deionised distilled water.

The following equipment is required:

- glass beakers with a minimum volume of 500 mL per snail (e.g. 2.5 L if five snails per replicate are used)
- 10 mL glass Petri dishes
- glass pipettes
- oxygen meter
- pH meter
- conductivity meter
- thermometer
- stereomicroscope
- climate chambers or temperature regulated room with temperature indicator

Test medium: water

Reconstituted (synthetic) water should be used as test medium and be composed of 2.5 g sea salt (e.g. Tropic Marin®) and 1.5 g sodium hydrogen carbonate ($NaHCO_3$), dissolved in 10 litres deionised water. The reconstituted water is prepared and stored in a aquarium with at least 200 L tank volume. Before use it has to be aerated for at least 24 hours and circulated by a water pump continuously to guarantee complete dissolution of all ingredients. Special care must be taken not to take water from water pipes made of copper, since the corresponding Cu concentrations may invoke acute snail mortality.

Test conditions

Water parameters should be as follows:

- temperature 20 ± 1°C
- pH: 8.0 ± 0.5
- oxygen concentration: > 6 mg/L
- oxygen saturation: > 60%
- conductivity: 770 ± 100 µS/cm

Test vessels should contain 1000 ml of reconstituted water per adult snail, and be covered with a perforated lid or with a net in order to prevent snails escaping. Water should be aerated through glass pipettes (Pasteur pipettes). Dissolved oxygen content should be kept above 60%, however the test vessels should be aerated as little as possible to avoid stripping of test chemicals. The water in each test vessel should be completely renewed at least three times per week (preferably synchronized with feeding interval). Water quality parameters (pH, dissolved oxygen content, conductivity, temperature and total nitrite content) should be measured before the water renewal in all test vessels. Additional measurements of ammonium and nitrate should be made if necessary. Constant photoperiods of 16 hours light to 8 hours darkness should be maintained.

Feeding of test animals

Feeding should preferably be done daily, but at least three times per week (at two day intervals) with organic lettuce (e.g. with slices calibrated at approx. 1 g per group of five snails per day). Finely ground commercial feed such as TetraPhyll® can also be used.

Test concentrations and replicates

Chemical stability analyses should be performed in the run-up to the test to check for stability of the test substance, as degradability may have implications for choice of test media and substance renewal intervals. Usually the test substance will be added with every renewal of the exposure water.

At least 5 concentrations and a control group with at minimum six replicates of five snails per concentration should be tested in a geometric series with a factor between concentrations not exceeding 2.2. In the run-up to the definitive test, the performance of range finding studies will help to select appropriate test concentrations. If a solubilizer has been applied, a solvent control using the same amount of solvent as in the treatments should be used. The amount of solvent in test concentrations should be as small as possible, not exceeding a maximum concentration of 0.01% (100 µl/l or 100 mg/l). Dimethyl sulfoxide (DMSO) is recommended but the selection of the appropriate solvent depends on the chemical constitution of the test substance. If ethanol or other readily biodegradable solvents are used, the maximum concentration should be 0.003% to avoid bacterial and fungal growth and biofilm development in the test vessels. Further information on appropriate organic solvents may be obtained from Jarosz and Weltje (2008).

**Biological methods**

Test organisms should be taken from a healthy breeding stock (i.e. without any sign of stress and parasitism, which is normally revealed by enhanced mortality and comparably poor fecundity checked against the maxima and minima of the annual reproductive cycle). Test organisms must be maintained in culture conditions (light, temperature, medium and feeding) similar to those of the laboratory breeding stock. Sexual maturity, which is normally obtained at a shell height between 21 and 24 mm (Zonneveld and Kooijman, 1989) should not have been fully reached, but almost. Indeed, pulmonates have developed specialised sections in the oviduct to store and nourish sperm after copulation for a period of weeks to months (Fretter and Graham, 1962). Therefore, snails should not be fully mature before the beginning of exposure to the toxicant, in order to allow the assessment of its potential effects on spermatogenesis. In order to meet this requirement, snails should exhibit shell heights in the range 18-22 mm (approx. age = 3 months) at the beginning of the test. Such snails will reach maturity and begin to reproduce during the test.

Size-calibrated snails should be randomly introduced in each replicate test vessel containing the exposure water. The test vessels should then be daily checked for abnormal test conditions (e.g. bacterial or fungal growth) or behaviour of test organisms (for example avoidance of water), while feeding the snails. Dead snails should be daily counted and withdrawn from the aquaria to prevent deleterious effects on other snails. Changes in snail density within a replicate should be accounted for in the calculation of the amount of provided food per test vessel, and in the calculation of fecundity.

Effects on reproduction are estimated based on both fertility (ability to produce fertilized eggs) and cumulative fecundity (cumulated number of eggs produced per individual). Fecundity should be daily measured by counting and collecting egg-clutches (using e.g. a razor blade) in each test vessel. The number of eggs per clutch should then be determined using a stereomicroscope, after each clutch has been isolated in a Petri dish filled with 10mL of reconstituted water. While determining the number of eggs per clutch, eggs should be carefully observed in order to assess fertilization failures: abnormal numbers (referring to controls) of empty eggshells might indicate an impairment of the female function, and

abnormal numbers (referring to controls) of non-fertilized eggs might indicate an impairment of male function. Non-fertilized eggs (diffuse and whitish nucleus, no motion) are easily distinguished from developing embryos (compact yellow embryo, very slow motions, as defined by Lalah *et al.*, 2007).

Hatching success (percent hatch per clutch) and time ($HT_{50}$) can be determined as additional endpoints for 30 clutches per treatment group, using the following procedure. Egg-clutches are maintained in conditions of light and temperature similar to those of the laboratory breeding stock during 35 days. The number of eggs remaining in each egg-clutch is then determined using a stereomicroscope. Obtained values are used to calculate the percent hatch per clutch. If the $HT_{50}$ is to be calculated, then the number of remaining eggs in the clutch should be regularly monitored (e.g. every other day) from the beginning of clutch exposure until day 35.

In case of serious fecundity impairment by the toxicant, it might be necessary to extend the test duration in order to ensure obtaining enough data to avoid a loss of power in the subsequent statistical tests.

**Data and reporting**

Validity criteria

Oxygen concentration in the water should not be below 60% of air saturation value at test temperature before water renewal.

The pH of the water should be in the basic measuring range. Because of shell decalcification, the pH must not fall below pH 6.5.

The concentration of the test substance should be measured to ensure that the substance concentration has been maintained over the test period.

Mortality in adult control snails should not exceed 20%.

Appropriate positive controls (e.g. 25 ng EE2/l and 250 ng TBT as Sn/l) have to be applied to demonstrate the responsiveness of the snails to EDCs (comp. vom Saal and Welshons, 2006).

At least 70% hatching success is required in the control if hatching rate is to be determined.

Treatment of results

When planning the test, it should be taken into consideration whether the aim is to calculate a NOEC/LOEC (by use of ANOVA or comparable nonparametric tests) or ECx values (by use of (non-)linear regression). The demand for replicates is higher if the ANOVA statistic is used whereas the (non-)linear regression generally demands more concentrations.

Endpoints to be evaluated statistically are mortality, time to first reproduction, cumulative fecundity per snail, and hatching rate. Data are recorded e.g. in a spreadsheet. The mean, and variability parameters such as standard deviation or standard error of the mean, are calculated for the previously cited endpoints.

ECx (effect concentration)

ECx values are calculated using an appropriate statistical model (e.g. logistic or Weibull function). An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation. To compute the $EC_{50}$ or any other ECx, the individual replicates should be used as input for the regression analysis.

NOEC/LOEC (No Observed Effect Concentration/Lowest Observed Effect Concentration)

If a statistical analysis is intended to determine a NOEC/LOEC value, a one-way analysis of variance (ANOVA) and a multiple comparison procedure (e.g. Dunnett's or Williams' test) is recommended for embryo numbers. The latter allows definition of the treatment concentration that characterizes significant differences for reproduction parameters compared to the control.

One-way analysis of variance is based on the assumptions that the observations are independent and normally distributed, and that the variance of the observations is homogeneous across all toxicant concentrations and the control. These assumptions should be checked prior to using the ANOVA. If data sets do not comply with these requirements the normality and the homogeneity of variances can be increased by the following transformation of data before testing (cf. section 6.5.4 for details):

- quantal responses expressed as a percentage (e.g. survival or mortality) should be arcsine-square root transformed,
- counts (e.g. numbers of produced eggs and clutches) should be square root transformed,
- continuous data (e.g. growth or weight measures) should be transformed to the natural logarithm.

Following transformation, data sets have to be tested again for normal distribution and homogeneity of variances. Gaussian distribution can be verified e.g. by the Kolmogorov-Smirnow normality test, whereas Bartletts's test represents a method for checking homogeneity of variance. In case data do not meet the assumptions for ANOVA, non-parametric procedures such as the Kruskal-Wallis-Test should be used. For the analysis of mortality (quantal data) the Fisher's exact test is recommended if the mortality of the individuals is independent. If individuals are living in the same test vessel, this assumption is violated. If data follow a monotone concentration response, a step-down trend test such as Cochran-Armitage and Jonkheer should be used to analyse quantal responses.

**Reporting requirements**

Laboratory reports should include the following information:

Testing site

- identification of the laboratory, dates of testing and key personnel involved

Test substance

- chemical identification of the substance (name, structural formula, CAS number, etc.) and organic solvent (if any) including purity, manufacturer, lot or batch number
- relevant physicochemical properties (e.g. water solubility, vapour pressure)
- analytical method for quantification of the test substance where appropriate
- appropriate negative and positive controls

Test species

- scientific name, source and culture conditions

Test conditions

- test procedure applied including photoperiod and light intensity, test design (e.g. test concentration used, number of replicates, number of snails per replicate)
- method of test substance pre-treatment, stock solution preparation and substance delivery system
- nominal test concentrations, details of sampling for chemical analysis of the test concentrations and the analytical methods by which the concentrations were analysed (including recovery and detection limit)
- information on the test conditions regarding pH, salinity, hardness, conductivity, temperature and dissolved oxygen concentration and saturation, nitrate, nitrite and ammonium concentrations
- information on feeding (e.g. source and type of food, quantity, feeding interval)
- compliance with validity criteria

Results

- references to chemical and statistical procedures (including assessment of the assumptions on which they are based)
- tabular and graphical (when appropriate) presentation of all measured and calculated endpoints
- tabular presentation of measured test concentrations including quality control samples
- NOEC/LOEC (including mean values, standard errors, error degrees of freedom and the least significant difference, or standard error of the difference) or ECx values (including the mathematical equation of the model used to estimate ECx values, the slope of the concentration response, confidence limits around the slope and the ECx values, assessment of goodness-of-fit and if possible assessment of appropriateness of the model applied) should be reported and p-values should be specified
- any deviation from the protocol should be accompanied by a discussion on possible impact on the study results.