

# **Final Report**

## **Baseline study on intersex in *Littorina littorea* with recommendations for biological TBT assessment criteria**

on behalf of

Ministry of Transport, Public Works and Watermanagement  
Directorate-General for Public Works and Watermanagement  
National Institute for Coastal and Marine Management / RIKZ  
NL-2500 EX Den Haag, The Netherlands

Ordernumber: 67011202



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## 1 Introduction

Butyltin compounds are used for a variety of applications, e.g. as biocides, stabilizers, catalysts and intermediates in chemical syntheses. Tributyltin (TBT) compounds exhibit the greatest toxicity of all organotins and have even been characterized as one of the most toxic groups of xenobiotics ever produced and deliberately introduced into the environment (Müller et al., 1989; Stewart et al., 1992). TBT is not only used as an active biocidal compound in antifouling paints, which are designed to prevent marine and freshwater biota from settlement on ship hulls, harbour and offshore installations, but also as a biocide in wood preservatives, textiles, dispersion paints and agricultural pesticides. Additionally, it occurs as a by-product of mono- (MBT) and dibutyltin (DBT) compounds, which are used as UV stabilizer in many plastics and for other applications.

Among the broad variety of malformations caused by TBT in aquatic animals (for review see Bryan & Gibbs, 1991; Fent, 1996), molluscs have been found to be one of the most sensitive groups of invertebrates. TBT-induced detrimental effects in Great Britain, Ireland and France, e.g. malformations of oyster shells and the imposex phenomenon of prosobranchs, were first noticed in the 1980s. Consequently, legislative restrictions were drawn up to reduce TBT concentrations in coastal waters and the application of TBT based paints was banned - with few exceptions - for all boats less than 25 m overall length. In autumn 2001, the International Maritime Organization (IMO) agreed on a total TBT ban for all antifouling paints by January 2003 and the presence of such paints on ship hulls by January 2008.

The imposex phenomenon of prosobranchs has been successfully used as a biological effect monitoring system to determine the degree of environmental TBT pollution. This biological marker allows also the assessment of ameliorations of the exposure situation following legislative controls. In most cases the dog whelk *Nucella lapillus* has been used as the sentinel species (e.g. Gibbs et al., 1987; Harding et al., 1992); considerations of additional species such as *Trivia arctica*, *Nassarius (Hinia) reticulatus*, *Ocenebra erinacea*, *Buccinum undatum* or *Neptunea antiqua*, were occasional exceptions in European surveys (e.g. Stroben et al., 1992a-c; Oehlmann et al., 1993, 1996a, b; Ten Hallers-Tjabbes et al., 1994).

*Nucella lapillus* as the established TBT effect monitoring species does not occur in many European coastal regions such as the southern part of the North Sea and the entire Baltic, mostly because of unsuited habitats. In addition, they become extinct near TBT point sources, like harbours and marinas, due to the existing level of TBT pollution. The need for a further effect monitoring system, which can be used in those areas, where dog whelks and other neogastropods are absent, was addressed by Bauer et al. (1993, 1995, 1997) and Oehlmann et al. (1994), who were the first to describe the intersex phenomenon in the periwinkle *Littorina littorea*. Intersex is characterized by a phenotypic disturbance of sex determination

between gonad and genital tract. Since the first description of intersex in periwinkles further investigations have been performed in France (Oehlmann, 1998), Ireland (Minchin et al., 1996, 1997) and Germany (Oehlmann et al., 1998b). These analyses have shown that *L. littorea* is well suited for TBT effect monitoring especially in regions where a relatively high level of contamination exists and that even here the species is very common and can be sampled in sufficient numbers because: a) it is tolerant of high TBT levels, b) it recruits from the plankton and c) it can occur in areas where dog whelks have expired. The intersex phenomenon was considered by OSPAR (Oslo and Paris Commissions) along with the imposex response in the dog whelk for use in biological TBT effect monitoring surveys in the entire convention area. The OSPAR guidelines for the TBT specific biological effect monitoring using the responses of both prosobranch species were amended recently (Oslo and Paris Commissions, 2001).

Within OSPAR, the Netherlands act as a lead country for the implementation of organotin compounds in the Joint Assessment and Monitoring Program (JAMP). At the ASMO meeting in Ostend, 26–30 March 2001, the Netherlands were asked to coordinate during 2001/2002:

1. the further revision of Technical Annex 3 on TBT-specific biological effects monitoring to the JAMP Guidelines for contaminant-specific biological effects monitoring;
2. the further development of the draft assessment criteria for TBT specific biological effects taking into account the comments made at this meeting and the advice of relevant experts.

This study aims to amend the assessment criteria for biological TBT effects in the dog whelk *Nucella lapillus*, which were presented at the SIME 2001 meeting. In extension to these already proposed assessment criteria in *N. lapillus*, a baseline study on intersex in the periwinkle *Littorina littorea* was performed to develop comparable assessment criteria on the background of the new EU Water Framework Directive (WFD). It was agreed to use a 5-step-approach for the development of intersex assessment criteria in *Littorina littorea*:

1. **Literature study:** The published literature on intersex in *Littorina littorea* should be screened using standard literature databases (Biological Abstracts, Current Contents, PubMed with MedLine, Zoological Records, etc.) and the main findings in these publications should be summarised. Special emphasis should be laid on the use of intersex as a biological effect marker in national or regional biological TBT effect monitoring surveys, on published information regarding concentration effect relationships and comparisons of the effects in *Littorina littorea* with those in the dog whelk *Nucella lapillus*.
2. **Research programme:** Given the case that the literature study reveals that the scientific basis for the development of assessment tools or criteria is not sufficient, a supplementary research

programme should be recommended to fill these gaps. Otherwise, the data originating from the literature study should be used for the further steps in the work programme.

3. **Calibration of biological markers in *Littorina littorea* and TBT:** The different measures of intersex intensities according to Oslo and Paris Commissions (2001), i.e. intersex index and average length of prostate gland in females, should be analysed with respect to their ecological and ecotoxicological relevance (for example loss of fertility) and in the sense of concentration effect relationships. Furthermore, an interspecific comparison between intersex in *Littorina* and imposex in *Nucella* should be made to derive species-specific differences in the TBT sensitivity.
4. **Evaluation of the results:** The results of the first three steps in the work programme should be evaluated. Special emphasis will have to be laid on the identification of threshold values for not acceptable ecological effects (occurrence of sterilised specimens in the populations, complete loss of the population's reproductive capability).
5. **Recommendations for biological assessment criteria:** A classification scheme based on the five different ecological status classes as defined by the European WFD will be proposed.

These five different steps in the approach will be addressed in the following chapters of the report.

## 2 Literature study

The following databases were screened for the published literature on:

- intersex investigations with *Littorina littorea* in the field and in the laboratory (search strings: "Littorina AND intersex"; littorinid AND intersex"; "\*winkle AND intersex"; "snail and intersex"),
  - other effects of organotin compounds, including those of TBT (search strings: "Littorina AND TBT AND effect"; littorinid AND TBT AND effect"; "\*winkle AND TBT AND effect"; "Littorina AND organotin AND effect"; littorinid AND organotin AND effect"; "\*winkle AND organotin AND effect"),
  - the use of *Littorina littorea* in biological TBT effect monitoring surveys (search strings: "Littorina AND TBT AND monitor\*"; littorinid AND TBT AND monitor\*"; "\*winkle AND TBT AND monitor\*"; "Littorina AND TBT AND indicat\*"; littorinid AND TBT AND indicat\*"; "\*winkle AND TBT AND indicat\*"; "Littorina AND organotin AND monitor\*"; littorinid AND organotin AND monitor\*"; "\*winkle AND organotin AND monitor\*"; "Littorina AND organotin AND indicat\*"; littorinid AND organotin AND indicat\*"; "\*winkle AND organotin AND indicat\*").
1. **Biological Abstracts** with two versions: "Biological Abstracts" and "Biological Abstract/RRM (Report, Reviews, Meetings)", both screened for the years 1994 to 2001.
  2. **Current Contents also** with two versions: "Agriculture, Biology & Environmental Science" and "Life Science", both screened for the years 1993 to 2001.
  3. **PubMed** with **MedLine**, which are available at the National Library of Medicine homepage (<http://www.ncbi.nlm.nih.gov/PubMed/>). This database includes records for the years 1965 to 2001.
  4. **"Web of Science"** at the Institute of Scientific Information (ISI) (homepage: <http://elib.tu-darmstadt.de/WoS/>). This programme allows a full search for articles by subject term, author name, journal title or author affiliation for the years 1994 to 2001.
  5. **Zoological Records**, published by the Zoological Society of London. The database was screened for the years 1993 to 2001.

Additionally, our own database with literature on TBT effects at different biological integration levels in molluscs was considered, which includes also reports from congresses, workshops and symposia. From the different sources a total of 94 references were extracted. 52 of these publications provided no data on *Littorina littorea* or other members of the family, but discussed their findings on the background of other reports on TBT effects in the periwinkle. Therefore, these articles were considered in the databases with reference to "Littorina" in the category "keyword plus" (or similar, depending on the database). The re-

maining 42 publications are summarised in the following (in chronological order of publication; within years in alphabetical order) including a rating of the publication regarding the applied biological measures as:

- A Relevant for the development of biological TBT assessment criteria and methodology of the cited study is identical with or exhibits only minor differences to the Technical Annex 3 on TBT-specific biological effects monitoring of the JAMP guidelines (Oslo and Paris Commissions, 2001).
- B Relevant for the development of biological TBT assessment criteria but other techniques and methods have been used than those proposed by Technical Annex 3 on TBT-specific biological effects monitoring of the JAMP guidelines (Oslo and Paris Commissions, 2001).
- C Irrelevant for the development of biological TBT assessment criteria.

Due to the fact that a variety of methods are available to determine TBT and other organotin compounds in water, sediments and snail tissues, a number of different analytical methods have also been considered in the cited literature, including graphite furnace (GF-) AAS following a selective extraction of compounds, GC-MS, GC-MS<sup>n</sup>, GC-ICP-MS, etc. Following the philosophy already expressed in the JAMP guidelines, it is not appropriate to specify one or more methods that should be considered whereas others have to be excluded. Therefore, all publications were considered, which met the requirements of the performance characteristics expressed in the JAMP guidelines, including (a) the specificity for TBT (rather than measuring total tin), (b) the concentration range from 0.05 – 1.6 mg/kg dry wt. (equivalent to 0.02 – 0.6 mg/kg wet wt.), (c) the detection limit of 0.02 mg/kg dry wt. (0.01 mg/kg wet wt.), and (d) quality assurance and control measures such as the parallel analysis of certified reference material (CRM).

**Carruesco et al. (1986)** investigated the tin contamination in *Littorina littorea* from the Bay of Arcachon in western France. Because they had no possibilities to analyse TBT in the tissues, the authors performed total tin measurements. The measured tin concentrations ranged from 600 to 7,900 µg Sn/kg dry weight in summer and from 100 to 2,200 µg Sn/kg in winter. Biological effects were not investigated in their approach. **Rating: C.**

**Langston et al. (1987)**, a publication which is almost identical with **Langston et al. (1990)**, analysed the TBT body burden in *Littorina littorea* populations from 9 sites near Poole Harbour in southern England. The measured concentrations ranged from 100 to 1,120 µg TBT as Sn/kg dry weight at ambient TBT concentrations from 2 to 139 ng TBT as Sn/l with resulting bioconcentration factors (BCFs) from  $8.1 \times 10^3$  to  $5.0 \times 10^4$ . Both publications report no effect data. **Rating: C.**



**Bryan & Gibbs (1991)** provide in their review on TBT effects in aquatic organisms some information on the bioaccumulation of this organotin compound in the periwinkle. They determined a mean body burden of  $404 \pm 171 \mu\text{g TBT as Sn/kg dry weight}$  ( $n = 4$ ) in populations from Northam Bridge (Itchen Estuary) at ambient TBT concentrations of  $27.3 \pm 16.2 \text{ ng as Sn/l}$  ( $n = 8$ ), resulting in a BCF of  $1.48 \times 10^4$ . The publication provides no information of biological TBT effects in periwinkles. **Rating: C.**

**Matthiessen et al. (1991)**, a publication which is almost identical with **Matthiessen et al. (1995)**, report size frequency and abundance variations in *Littorina littorea* in the estuaries of the rivers Crouch (Essex) and Hamble (Hampshire) between 1986, i.e. one year before the partial ban of TBT-based paints in the U.K., and 1991. In the first three years of the survey, the most TBT contaminated stations of the Crouch estuary were characterised by a predominance of adult periwinkle specimens (shell height  $> 12 \text{ mm}$ ) and an almost complete lack of younger snails. In the following years, the abundance of subadult *Littorina* specimens in the populations increased slowly with a parallel decrease of TBT residues in water, sediments and snail tissues. In the even more contaminated Hamble estuary no comparable signs of a population recovery could be assessed until 1991, while in the Blackwater estuary, which was chosen as a reference area, the size frequency in *Littorina* populations was intact without any indication for a shift in favour of adult specimens at any time of the survey. Furthermore, plankton surveys of the Crouch estuary showed that the numbers of *Littorina littorea* eggs and veliger larvae have progressively increased since the TBT restrictions, suggesting that TBT may have impaired periwinkle reproduction and/or survival of the eggs and larvae. The authors conclude that a reduction of egg production was the most probable mechanism of action due to the fact that periwinkles do not develop imposex and because they could show in own laboratory experiments that egg production was significantly affected in TBT-treated animals. With regard to the special objective of the current study, Matthiessen et al. (1991, 1995) provide valuable information on TBT concentrations in the Crouch between 1986 and 1990, which allow deriving effect concentrations for an impairment of reproduction in periwinkles. During the phase of poor population recruitment the maximum TBT concentrations in the Crouch were  $45 \text{ ng/l}$ , in the later phase of populations recovery the concentrations attained maximum values of  $7 \text{ ng/l}$ . **Rating: B.**

**Bauer et al. (1993)** is an extended report of a research project conducted in summer 1993 to assess TBT effects in *Littorina littorea* populations at 11 stations on the German North Sea coast. The publication is the first description of intersex and provides already the detailed morphological features of this TBT response, including the intersex development scheme. Further possible effects on the morphological and histological level are described and measured TBT residues in the tissues of the snails reported.. The publication provides a preliminary body burden effect relationship for the 11 analysed populations and a puta-

tive threshold concentration for the induction of intersex in the range of 15 ng TBT as Sn/l. A short summary of these results was published later by **Brumm-Scholz et al. (1994)**. **Rating: A.**

**Brick & Deutsch (1993)** identified in a comparative ultrastructural study cell structure changes in penis epithelia of a number of prosobranch species, which were attributed as a direct consequence of TBT exposure of snails in their natural environment. The authors found the most drastic effects in *Ocenebrina aciculata*, followed by *Hinia reticulata*, *Hinia incrassata* and finally *Littorina littorea*. These findings were reported to be in line with the species-specific TBT sensitivities in the prosobranchs if imposex analyses as measures at the organism level are considered. Because intersex as a specific TBT response in periwinkles was not known at the time of manuscript acceptance, the attempt of Brick & Deutsch (1993) does not provide any measures of intersex intensities or of analytically measured TBT concentrations in the analysed snail populations. **Rating: C.**

**Deutsch & Brick (1993)** investigated TBT-induced pathological changes in epithelia of the penis, the sperm groove and the prostate in male *Littorina littorea* sampled at different sites near Roscoff (Brittany). The results were compared with those for the imposex-affected neogastropod species *Ocenebrina aciculata* living sympatrically with the periwinkle but exhibiting an even higher sensitivity than *Nucella lapillus*. The authors conclude that the comparatively lower TBT sensitivity of periwinkles can also be confirmed at the level of ultrastructural cell changes. **Rating: C.**

**Kure & Depledge (1993)** determined organotin concentrations in sea water, sediments and selected molluscs, including *Littorina littorea* in Danish coastal waters. The degree of organotin contamination varied with the proximity of sampling stations to marinas and other harbours. Seasonal changes in TBT concentrations coincided with removal of yachts from harbours during winter. *Littorina littorea* exhibited BCFs of 500 to 10,000 at marina sites and up to 22,000 away from these point sources. No biological effect measures were considered in this study. **Rating: C.**

**Oehlmann et al. (1994)** is a short summary of the results of the basic study conducted by Bauer et al. (1993) on the German North Sea coast with the first description of the intersex phenomenon in *Littorina littorea* on the occasion of an international littorinid congress. Later, the findings were published in a regular article (Bauer et al., 1995). Therefore the current report will refer to the original work and the extended English publication instead of the abstract version. **Rating: C.**

**Bauer et al. (1995)** summarise the results for measured TBT residues, intersex intensities and histopathological alterations in the midgut gland and ovary of *Littorina littorea* samples from the German North Sea coast in 1993. The publication provides a preliminary body burden effect relationship for the 11 ana-

lysed populations and a putative threshold concentration for the induction of intersex in the range of 15 ng TBT as Sn/l. **Rating: A.**

**Deutsch & Fioroni (1996)** studied the effects of TBT exposure on imposex development and on the size of the accessory female gland complex in *Littorina littorea*. For this purpose, field studies were performed in parallel in Roscoff (Brittany) and Helgoland (North Sea) with an injection of TBT into the soft tissues of the snails and additionally aqueous exposure experiments in the laboratory. The authors could not induce imposex, what is not surprising as the periwinkle does not exhibit this TBT response. On the other hand, they did not look for malformations of the female gland complex in periwinkles so that a potential intersex development was simply missed in this study. The authors report a single “imposex-affected” specimen in the sampled Helgoland population but this snail was later identified as an intersex stage 4 female with penis development. In all TBT exposed field and laboratory groups the size of the female glandular complex was significantly reduced compared to the controls. **Rating: C.**

**Deutsch et al. (1996)** performed a number of field and laboratory TBT experiments with *Littorina littorea*. The authors report that the species does not exhibit imposex, but that an exposure to the organotin compound causes a reduction of the female glandular complex and affects also the penis length of male specimens. **Rating: C.**

**Minchin et al. (1996)** conducted a survey of biological TBT effects in the region of Cork harbour on the south coast of Ireland, using imposex in *Nucella lapillus* and intersex in *Littorina littorea* populations as parameters simultaneously. Due to their higher sensitivity, dog whelks had become extinct in some areas of the harbour since 1968, while *Littorina* tolerates lower salinities, is less affected by TBT, and was therefore used to monitor in more brackish and heavily contaminated areas. Both measures, imposex and intersex intensities, were consistent in terms of the pattern of TBT contamination in Cork harbour. The results from this study were re-evaluated by Oehlmann et al. (1998a) for the interspecific comparison of imposex and intersex. **Rating: A.**

**Ronis & Mason (1996)** investigated the effects of an in vivo and in vitro exposure of *Littorina littorea* to TBT on the ability of the snails to metabolise testosterone. The authors describe a marked reduction of phase II metabolism of testosterone, resulting in a decrease of testosterone excretion and a consequent increase of endogenous androgen concentrations in TBT exposed periwinkles. Additionally, a reduction of the aromatase activity as a phase I enzyme by 30-40% was found. The authors conclude that the major molecular target of TBT in *L. littorea* is the phase II metabolism of androgens and not the phase I enzymes as described for imposex affected prosobranchs. Intersex intensities were not analysed in the study

of Ronis & Mason (1996). Consequently, no relevant information for the development of biological TBT assessment criteria can be taken from this publication. **Rating: C.**

**Bauer et al. (1997)** present in their study the most important *Littorina* data from the German TBT survey in 1994/95, which are documented on a broader basis in Oehlmann et al. (1998b). Additionally the results from laboratory experiments are communicated providing evidence for a successive loss of TBT sensitivity during ontogenetic development of periwinkles. TBT is capable to induce intersex only in young, sexually immature snails. In adult periwinkles even high TBT exposure does not result in the development of intersex or an enhancement of the already established intersex intensities. These findings have been considered for the JAMP guidelines with a selective sampling of certain age and size classes of *L. littorea*. **Rating: A.**

**Minchin et al. (1997)** analysed the intensities of imposex in *Nucella lapillus* and of intersex in *Littorina littorea* populations in Donegal Bay on the northwest coast of Ireland to monitor the degree of TBT contamination in this area with the fishing port of Killybegs being the main source of contamination. Dogwhelks had become extinct since 1987 in the harbour area and the remaining populations near the harbour mouth were so seriously affected by imposex development that they were likely to die out in the future. In these highly contaminated areas periwinkles were still present and their intersex condition was used as a biomarker of TBT exposure and effects. Both indicator species complimented each other. The results from this study were re-evaluated by Oehlmann et al. (1998a) for the interspecific comparison of imposex and intersex. **Rating: A.**

**Bauer (1998)** gives an extended overview of TBT effects in periwinkles, based on laboratory and field investigations, and evaluates the use of the species as a TBT bioaccumulation indicator as well as its potential for biological TBT effect surveys. The thesis provides data on intersex intensities, measured as ISI values, mean length of the prostate gland in females and percentage of sterilised females, but also on TBT effects in males (e.g. percentage of males with penis malformations, mean number of penial glands) and correlates these effects with measured TBT concentrations in sediments and snail tissues. Furthermore, biomonitoring results are presented for Germany and Ireland and also for a limited number of French populations. Due to its character as a thesis, many of the reported results had already been published earlier (e.g. Bauer et al., 1993, 1995, 1997; Minchin et al. 1996, 1997; Oehlmann et al., 1998b). **Rating: A.**

**Casey et al. (1998)** used intersex in *Littorina littorea* populations to monitor the degree of TBT pollution in the Cork harbour area in southern Ireland. The response was used for the first time in Ireland as a medium-term indicator of TBT pollution and results showed that there were no deviations in intersex levels over the three years period from 1994 to 1996. **Rating: A.**

**Matthiessen & Gibbs (1998)** reviewed the field and laboratory evidence for TBT-induced endocrine disruption in molluscs. Imposex in prosobranch snails and intersex in *Littorina littorea* are rated as the clearest examples for endocrine disruption in invertebrates, which both are caused by elevated testosterone titres in the snails following an exposure to TBT. Although the precise mechanism has not yet been fully described, the weight of evidence suggests that TBT acts as a competitive inhibitor of the cytochrome P-450-mediated aromatase. **Rating: C.**

**Oehlmann (1998)** reports in his comparative approach on the use of different biological TBT effects in prosobranch snails, including imposex and intersex, for biological effect monitoring purposes. The various parameters for the measurement of intersex intensities are evaluated with regard to their ecological relevance, sensitivity and robustness. The study provides detailed information on TBT bioaccumulation in periwinkles and also on concentration response relationships, based on measured TBT concentrations in ambient water and body burdens. An intercalibration of the specific TBT responses is performed for a number of prosobranch snail species and possibilities and limitations of the use of these snails for spatial and temporal trend monitoring purposes are presented with case studies for France, Germany and Ireland. **Rating: A.**

**Oehlmann et al. (1998a)** compared imposex in *Nucella lapillus* and intersex in sympatrical populations of *Littorina littorea* in different European countries. The main objective of this work was to evaluate the geographical uniformity of both TBT responses, to provide detailed information on concentration effect relationships and to intercalibrate the two biomonitoring. The authors could show that the dog whelk is the better suited species for only slightly or moderately TBT contaminated areas (ambient concentrations < 2.0 ng TBT as Sn/l), while the assessment of intersex intensities in periwinkle populations has considerable advantages in regions with higher TBT concentrations. These findings were already considered for the JAMP guidelines. **Rating: A.**

**Oehlmann et al. (1998b)** have performed an extended research and development project with the main objective to calibrate TBT-specific biological responses in the periwinkle for biological effect monitoring programmes. Special emphasis was laid on intersex and histopathological alterations in different target organs. This report led to the implementation of intersex as a second TBT effect next to the imposex response in *Nucella lapillus* for the JAMP guidelines. The results were also published in a number of review articles on TBT effects in prosobranchs and on the possibilities and limitations of biomonitoring approaches (e.g. **Schulte-Oehlmann et al., 1996; Oehlmann et al., 1996a, 1997a, b, 1999**). **Rating: A.**

**Schulte-Oehlmann et al. (1998)** analysed imposex in *Hydrobia ulvae* and intersex in sympatrical populations of *Littorina littorea* in a comparative approach to evaluate the species-specific sensitivity of both TBT induced virilisation phenomena. The study concludes that the mud snail is the more sensitive bioindicator when compared with periwinkles. Because *H. ulvae* is not considered as a monitoring species for the JAMP, the data provided by Schulte-Oehlmann et al. (1998) are not relevant for the development of biological TBT assessment criteria in *L. littorea*. **Rating: C.**

**Sundermann et al. (1998)** compared the ultrastructure of prostate gland cells in males and intersex stage 3 females of *Littorina littorea* and found no differences regarding form, structure, organelles and secretion products. These findings demonstrate that in intersex stage 3 females the epithelium of the pallial oviduct section, which originally consists of different glandular parts, is gradually transformed into a male prostate gland. **Rating: C.**

**Ide & Watermann (1999)** republished the results of Bauer et al. (1993, 1995) as a contribution to the common environmental status report for the German wadden sea of the German Federal Environmental Agency and the National Park Wadden Sea. We will use the original data for the current report. **Rating: C.**

**Matthiessen et al. (1999)** reviewed the published literature for existing evidence of endocrine disruption in invertebrates. The case study of organotin effects in molluscs, especially imposex in prosobranchs and intersex in *Littorina littorea*, is rated as the best documented example in wildlife animals. The information on intersex in periwinkles, particularly threshold concentrations for the induction of intersex and the occurrence of sterilised females, were taken from Oehlmann (1998). Therefore the current report will refer to the original studies instead of the review article. **Rating: C.**

**Morcillo et al. (1999)** utilised mussels as sentinels of environmental pollution with TBT and triphenyltin (TPT) compounds on the Catalan coast in Spain and determined residue levels for both compounds and their metabolites in the mussel tissues. Additionally, the authors performed measurements of the cytochrome P-450-dependent aromatase in mussels from these different sites and found evidence for a TBT-induced inhibition of this enzyme. It is concluded that TBT shows the identical biochemical mechanism on steroid metabolism in mussels and imposex and intersex affected snails, such as *Littorina littorea* (referring to the work of Ronis & Mason, 1996). **Rating: C.**

**Nehring (1999)**, a publication which is almost identical with **Nehring (2000)**, reviews the effects of TBT on snail populations with special emphasis of report from the German North Sea coast. The author provides no new data for any of the considered species, including *Littorina littorea*, but refers to the work

and results of Bauer et al. (1995, 1997), Brumm-Scholz et al. (1994), Oehlmann et al. (Oehlmann et al., 1997a, b, 1998 a, b) and Schulte-Oehlmann et al. (1996, 1998). His main conclusion is that at least for the prosobranch species *Buccinum undatum*, *Hydrobia ulvae*, *Littorina littorea* and *Nucella lapillus* significant ecological effects of TBT pollution are very probable. **Rating: C.**

**Nohara (1999)** examined the anatomy of the Pacific littorinid species *Littorina sitkana*, a species closely related to *Littorina littorea*, from 9 localities in northern Japan. In contrast to the Atlantic species, *L. sitkana* exhibited imposex at a number of stations, which were likely to be exposed to TBT, indicating marked differences in the TBT responses within the family Littorinidae. The imposex intensity and thus the TBT sensitivity in *L. sitkana*, measured by the length of the penis in females, was lower than in sympatrically living *Nucella* populations in Japan. The publication, although interesting from a general ecotoxicological perspective, is irrelevant for the main objective of the current study. **Rating: C.**

**Barroso et al. (2000)** conducted a survey of TBT contamination in the Ria de Aveiro estuary in north-west Portugal, using the imposex response in three gastropod species (*Nucella lapillus*, *Nassarius reticulatus*, *Hydrobia ulvae*) and intersex in *Littorina littorea*. A total of 45 stations were sampled for gastropods from May to July 1998 and at 17 of these stations TBT concentrations were determined in sea water. The results indicate that *Nucella lapillus* was the most sensitive species to TBT, followed, in decreasing order, by *Nassarius reticulatus*, *Hydrobia ulvae* and *Littorina littorea*. The intersex index in the study area ranged from 0.3 to 0.5. **Rating: A.**

**Wappelhorst et al. (2000)** analysed intersex intensities and measured residues of butyl- and phenyltin compounds in 10 periwinkle populations from the German North Sea and Baltic coast according to the JAMP guidelines. The stations sampled for *Littorina littorea* are part of the German "Bund-Länder-Messprogramm" and reflect background contamination levels of the German North Sea and Baltic coast. The authors propose for the first time biological TBT assessment criteria for intersex in *Littorina littorea* considering the five ecological status classes of the new EU Water Framework Directive. **Rating: A.**

**De Wolf et al. (2001)** investigated the degree of TBT contamination in the polluted Western Scheldt estuary in the Netherlands by using the intersex response in autochthonous *Littorina littorea* populations. As could be expected, intersex intensities, assessed by the intersex index (ISI; according to the JAMP guidelines) and by percentage of sterilised females, differed significantly between sites with highest values of up to 1.26 for the ISI and 33% sterilised females in the vicinity of the harbours of Antwerp and Vlissingen. Unfortunately, the authors did not analyse TBT concentrations in water, sediment or in the tissues at the sampled stations. **Rating: A.**

**Brozek & Brinch (2001)** summarise the Danish efforts to assess the environmental quality status of the Wadden Sea by the national Monitoring and Assessment Programme 2000. They report on the occurrence of intersex and sterility in Danish *Littorina littorea* populations but give no details regarding the range of measured ISI values, sterility incidences or measured TBT concentrations in ambient water, sediments or snail tissues. **Rating: C.**

**Korhammer et al. (2001)** analysed the intersex intensities in 5 periwinkle populations from the German North Sea coast and in 10 populations from the German part of the Baltic according to the JAMP guidelines. Additionally, the residues of butyl- and phenyltin compounds were determined in 53 sediments and evaluated toxicologically. The measured intersex intensities were assessed using the criteria already proposed by Wappelhorst et al. (2000). **Rating: A.**

The relevant information of all publications rated to the categories "A" and "B" is summarised in table 1.

Tab. 1. Summary of relevant information from positively evaluated publications (categories "A" and "B") in the literature study. Abbreviations: con. = concentration; sed. = sediments.

| Publication                    | Rating | TBT accumulation from |      | TBT effects |       | Con. response relationships |      | Threshold con. |      | Inter-calibration | Assessment criteria |
|--------------------------------|--------|-----------------------|------|-------------|-------|-----------------------------|------|----------------|------|-------------------|---------------------|
|                                |        | water                 | sed. | intersex    | other | water                       | sed. | water          | sed. |                   |                     |
| Matthiessen et al. (1991)      | B      |                       |      |             | X     |                             |      | X              |      |                   |                     |
| Bauer et al. (1993)            | A      |                       |      | X           | X     |                             |      | X              |      |                   |                     |
| Brumm-Scholz et al. (1994)     | A      |                       |      | X           | X     |                             |      | X              |      |                   |                     |
| Bauer et al. (1995)            | A      |                       |      | X           | X     |                             |      | X              |      |                   |                     |
| Matthiessen et al. (1995)      | B      |                       |      |             | X     |                             |      | X              |      |                   |                     |
| Minchin et al. (1996)          | A      |                       |      | X           |       |                             |      |                |      | X                 |                     |
| Oehlmann et al. (1996a)        | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Schulte-Oehlmann et al. (1996) | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Bauer et al. (1997)            | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Minchin et al. (1997)          | A      |                       |      | X           |       |                             |      |                |      | X                 |                     |



| Publication               | Rating | TBT accumulation from |      | TBT effects |       | Con. response relationships |      | Threshold con. |      | Inter-calibration | Assessment criteria |
|---------------------------|--------|-----------------------|------|-------------|-------|-----------------------------|------|----------------|------|-------------------|---------------------|
|                           |        | water                 | sed. | intersex    | other | water                       | sed. | water          | sed. |                   |                     |
| Oehlmann et al. (1997a)   | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Oehlmann et al. (1997b)   | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Bauer (1998)              | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Casey et al. (1998)       | A      |                       |      | X           |       |                             |      |                |      |                   |                     |
| Oehlmann (1998)           | A      | X                     | X    | X           | X     | X                           |      | X              |      | X                 |                     |
| Oehlmann et al. (1998a)   | A      |                       |      | X           |       |                             |      |                |      | X                 |                     |
| Oehlmann et al. (1998b)   | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Oehlmann et al. (1999)    | A      |                       | X    | X           | X     |                             | X    | X              | X    |                   |                     |
| Barroso et al. (2000)     | A      |                       |      | X           |       |                             |      |                |      | X                 |                     |
| Wappelhorst et al. (2000) | A      |                       |      | X           | X     |                             |      |                |      |                   | X                   |
| De Wolf et al. (2001)     | A      |                       |      | X           |       |                             |      |                |      |                   |                     |
| Korhammer et al. (2001)   | A      |                       |      | X           | X     |                             |      |                |      |                   | X                   |

### 3 Research programme

The results of the literature study reveal a number of studies with valuable data for the development of biological TBT assessment criteria for intersex in *Littorina littorea*. Even two publications exist, which propose assessment criteria with five different ecological status classes according to the new EU Water Framework Directive. Nevertheless, there are two major shortcomings in the published intersex data:

1. Although it has been shown that next to TBT also triphenyltin (TPT) compounds are able to induce imposex in at least two prosobranch snail species, the rock shell *Thais clavigera* (Horiguchi et al., 1995, 1997) and the ramshorn snail *Marisa cornuarietis* (Schulte-Oehlmann et al., 2000) with negative results for the European species *Nucella lapillus* and *Nassarius reticulatus* (Bryan et al. 1988, Schulte-Oehlmann et al., 2000), no comparable investigations have been performed with *Littorina littorea* so far. Wappelhorst et al. (2000) demonstrated that TPT residues in the tissues of periwinkles exceed generally TBT tissue concentrations in North Sea areas of background TBT exposure, although the snails exhibit very low intersex intensities indicating that also TPT might be a causative agent for the virilisation of females.

There is also a lack of information regarding the intersex inducing capacities of other environmentally relevant organotin compounds such as monobutyltin (MBT), dibutyltin (DBT), monophenyltin (MPT) and diphenyltin (DPT), which occur as degradation products of TBT and TPT. Bryan et al. (1988) and Horiguchi et al. (1997) report a negative result for these compounds in the imposex-affected prosobranchs *Thais clavigera* and *Nucella lapillus*, but no tests have been performed with periwinkles so far.

2. Oehlmann (1998) is the only report of a concentration effect relationship on the basis of TBT concentrations in ambient water and imposex intensities. This gap is not too astonishing as TBT concentrations in coastal waters are known to exhibit marked seasonal and even daily changes due to the tidal variation of water levels (Waldock et al., 1987; Hall, 1988). Consequently, most investigations have focussed on the determination of organotin residues in sediment samples and biota tissue instead of water analyses, which require a highly standardised sampling to provide representative results for a given station.

Both aspects should be considered in future research programmes but they do not hinder the development of assessment criteria because the intersex response, measured by the intersex index, exhibits a marked and statistically highly significant correlation to the percentage of sterilised females in the populations as a

parameter of extraordinary ecological relevance (cf. next section). Furthermore, the intercalibration of intersex in *Littorina littorea* and imposex in *Nucella lapillus* with its by far better data base for an aqueous TBT concentration effect relationship facilitates a prediction of attained intersex intensities in a periwinkle population at a given TBT concentration (chapter 4).

## 4 Calibration of biological markers in *Littorina littorea* and TBT

The Technical Annex 3 for the TBT specific biological effects monitoring of the OSPAR guidelines within the JAMP (Oslo and Paris Commissions, 2001) differentiates two parameters for the determination of intersex intensities:

1. the intersex index (**ISI**), calculated as the mean value of intersex stages within a population ( $ISI = \text{sum of intersex stage values of all females sampled} / \text{number of females}$ ) as the **primary index**. Females in the intersex stages 2, 3 and 4 are unable to reproduce due to oviduct malformations or the supplant of female sexual glands by the corresponding male formations. Females in the intersex stages 0 and 1 are rated as fully capable for normal reproduction although some evidence exists that stage 1 specimens are already characterised by a reduced reproductive success (cf. Bauer et al., 1993, 1995 for details). Therefore, ISI values  $> 1.00$  indicate that at least some of the females in the sample are sterilised due to intersex development. The ISI is the equivalent to the VDSI (vas deferens sequence index) in imposex affected prosobranch species such as *Nucella lapillus*.
2. the average female prostate length (**FPrL**), calculated as the mean length of the prostate gland in female periwinkles in a population ( $FPrL = \text{sum of prostate lengths of all females sampled} / \text{number of females}$ ) as the **secondary index** to allow a better differentiation of TBT exposure and effects in highly contaminated areas, when the ISI tends to attain its maximum value. A prostate gland is not developed before stage 3 of intersex development. Therefore, the FPrL attains values above 0 with the first occurrence of intersex stage 3 females in a sample and exhibits therefore a lower sensitivity compared with the ISI. The FPrL is the equivalent to the RPSI (relative penis size index) in imposex affected prosobranch species such as *Nucella lapillus*.

Figure 1 provides a conclusion of the relationship between the percentage of sterilised females in populations of *Littorina littorea* and the two intersex parameters according to the JAMP guideline. The correlations are based on the raw data from all publications summarised in table 1. The sterilisation of females is a toxicological endpoint of high ecological relevance because any impairment of reproduction causes an elevated risk for a consequent extermination of populations.

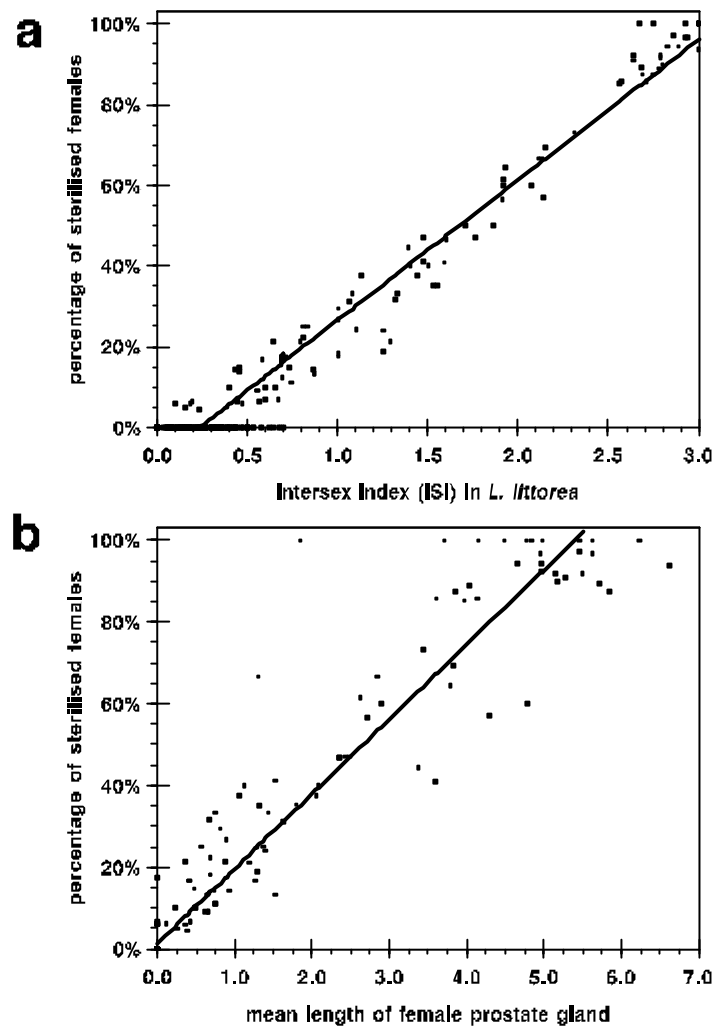


Fig. 1. *Littorina littorea*. Relationship between the incidence of sterile females and the intersex index (ISI) (a) or the mean female prostate length (FPrL) (b) with calculated correlations. Raw data were extracted from all publications summarised in table 1. (a)  $y = 34.7x - 8.09$ ,  $n = 268$  samples from 189 stations,  $r = 0.982$ ,  $p < 0.005$ . (b)  $y = 18.3x + 1.39$ ,  $n = 262$  samples from 152 stations,  $r = 0.765$ ,  $p < 0.005$ .

A direct comparison of the correlations between the percentage of sterilised females and ISI (Fig. 1a) or FPrL values (Fig. 1b) demonstrates that the ISI is the more robust parameter exhibiting a better correlation and also an optimal differentiation of TBT effects on the reproductive capability of snail populations. The data analysis shows that first sterile periwinkles may be found in a given population when the ISI attains a value of 0.10 and can be expected at ISI values  $> 0.30$ . Above values of 0.70 at least some females will be definitively sterilised in all analysed populations and virtually all females have ceased breeding at values above 2.50 (Fig. 1a). Contrary to the ISI correlation, up to 18% of females in a given periwinkle population can already be sterilised at FPrL values of 0 and up to 100% of all females at values of only 2.00, that means before this parameter attains 30% of its maximum (Fig. 1b). This direct comparison of both parameters shows that the ISI is the more suited measure of the intersex intensity, which has al-

ready been considered in the JAMP guideline. Therefore, concentration effect relationships and an inter-calibration with imposex parameters in *Nucella lapillus* will be performed in the following only for the ISI.

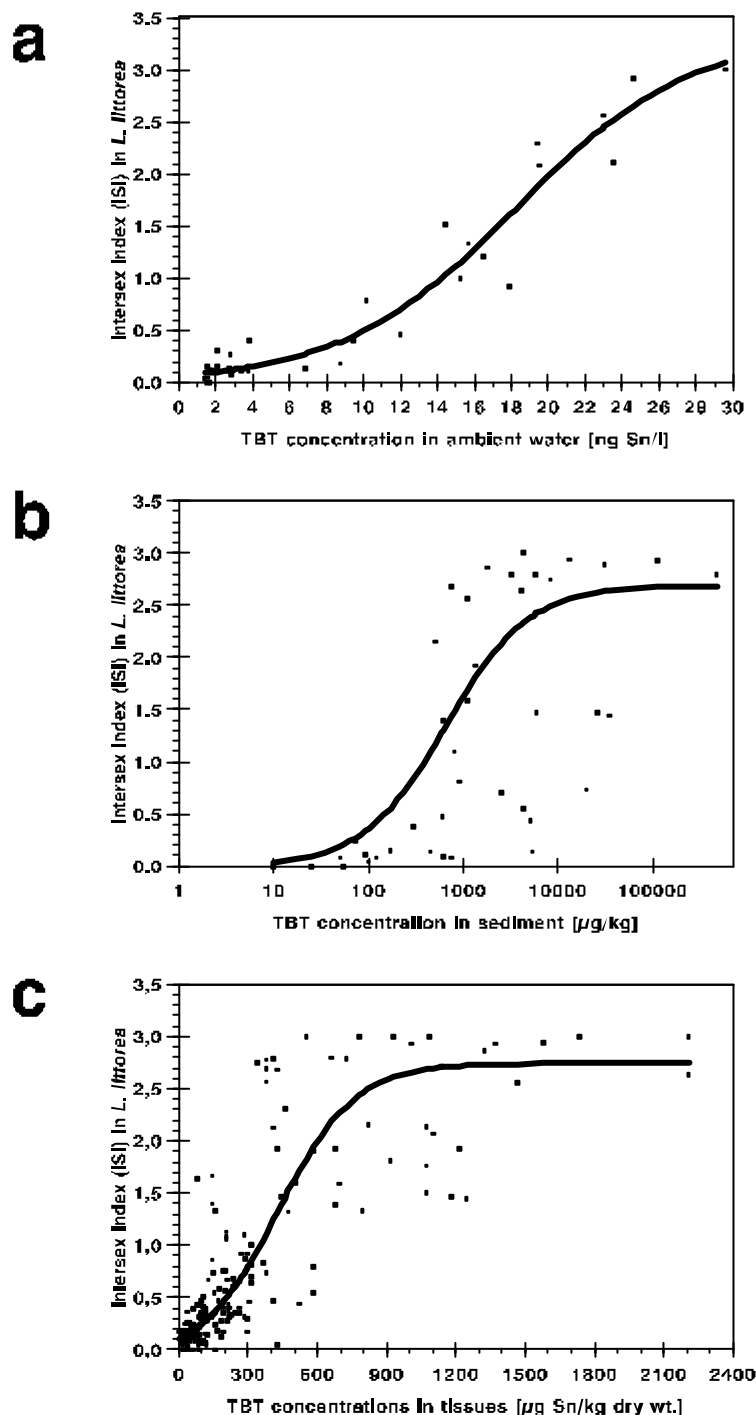


Fig. 2. *Littorina littorea*. Concentration effect relationships for the ISI, based on measured TBT concentrations in ambient sea water (a), sediments (b) and snail tissues (c). Raw data were extracted from all publications summarised in table 1. (a)  $y = 3.35 / (1 + e^{-(x - 18.3) / 4.73})$ ,  $n = 34$  samples from 27 stations,  $r = 0.975$ ,  $p < 0.005$ . (b)  $y = (2.69 x) / (652 + x)$ ,  $n = 40$  samples from 19 stations,  $r = 0.652$ ,  $p < 0.005$ . (c)  $y = 2.85 / (1 + e^{(-0.006(x + 427))}) - 0.105$ ,  $n = 189$  samples from 89 stations,  $r = 0.778$ ,  $p < 0.005$ .

The concentration response relationships for the ISI in *Littorina littorea* are presented in figure 2, based on measured TBT concentrations in ambient sea water (Fig. 2a), sediments (Fig. 2b) and snail tissue (Fig. 2c), respectively. ISI values of 0.10, normally indicating the absence of sterilised females in the populations, are likely to occur at TBT concentrations as low as 2 ng as Sn/l in water, 20 µg/kg in sediments and 40 µg as Sn/kg in snail tissues. The average threshold ISI value of 0.30 for the presence of first sterile females is attained at TBT concentrations of 6 ng as Sn/l in water, 80 µg/kg in sediments and 150 µg as Sn/kg in snail tissues. The critical ISI value of 2.50 for the virtually total loss of the reproductive capability in periwinkle populations is found at TBT concentrations of 23 ng as Sn/l in water, 8000 µg/kg in sediments and 800 µg as Sn/kg in snail tissues. These statistically derived data are in line with the observations reported by Matthiessen et al. (1991, 1995) for English *Littorina* populations reporting poor recruitment at ambient TBT concentrations in sea water of 45 ng/l (equivalent to 18.4 ng as Sn/l) and a recovery of the populations following a decrease of TBT concentrations below 7 ng/l (equivalent to 2.9 ng as Sn/l) after legislative restrictions.

The comparison of the aqueous TBT concentration ISI relationship in *Littorina littorea* with the correlation of ambient TBT concentrations and imposex intensity in *Nucella lapillus* (cf. Oehlmann et al., 1998a) reveals that the dog whelk is by far more sensitive. The threshold concentration for imposex induction is 0.5 ng TBT as Sn/l in *Nucella* (Gibbs et al., 1987) while the corresponding value for intersex induction in *Littorina* is 2.0 ng TBT as Sn/l (cf. Fig. 2a). Dog whelk females are sterilised at ambient TBT concentrations > 2.0 ng as Sn/l with a complete loss of their reproductive capability at > 10 ng as Sn/l, while the corresponding values are 6 and 23 ng as Sn/l for periwinkles.

The species-specific differences of the TBT sensitivity were also confirmed in monitoring surveys in the field, whenever both prosobranchs were used simultaneously to assess the degree of TBT contamination in a given area (e.g. Minchin et al., 1996, 1997; Oehlmann et al., 1998a; Barroso et al., 2000). These data are summarised in figure 3. At VDSI values ≤ 4.00 in dog whelk populations and ambient TBT concentration < 2.0 ng as Sn/l all females are still capable to reproduce, while the ISI in sympatrically living periwinkle populations is characterised by a kind of background noise with values ranging from 0 to 0.40, but normally not above 0.30. At highly contaminated sites with TBT concentrations > 2.0 ng as Sn/l, the VDSI exceeds 4.00 and *Nucella* populations become endangered due to progressive female sterilisation. It is in this contamination range that ISI values begin to increase. Should periwinkle populations exhibit an ISI of 0.50 or more, all females in a sympatrically living dog whelk population will have become sterile and the *Nucella* population will soon expire. In areas, where *Littorina* attains ISI values above 1.0 no living dog whelk populations have been reported in the available literature.

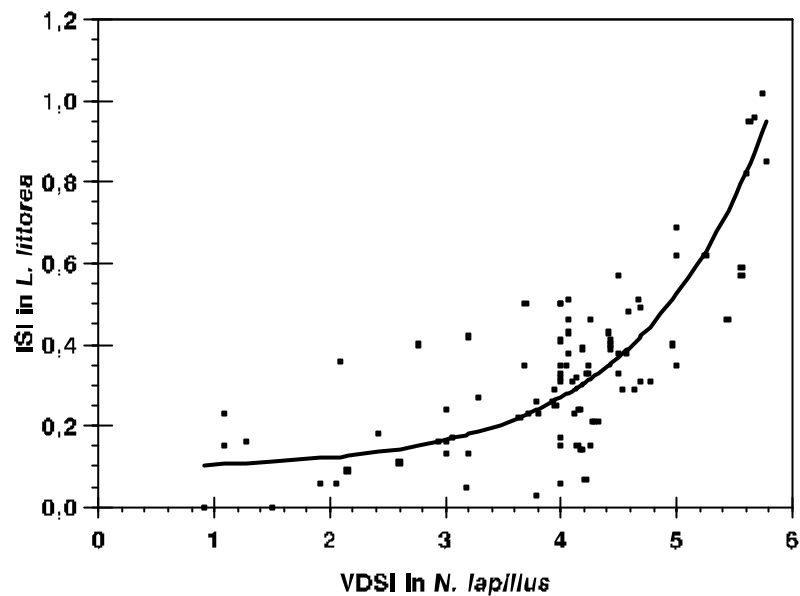


Fig. 3. Relationship between the intersex index (ISI) in *Littorina littorea* and the vas deferens sequence index (VDSI) in sympatrically living populations of *Nucella lapillus* (data from Minchin et al., 1996, 1997; Oehlmann et al., 1998a; Barroso et al., 2000) with calculated correlation:  $y = 0.132 + 0.0039 e^{(0.913 x)}$ ,  $n = 92$  samples from 91 stations,  $r = 0.814$ ,  $p < 0.005$ .

These species-specific differences regarding the TBT sensitivity of *Littorina* and *Nucella* will have to be considered for the development of biological assessment criteria for intersex intensities in periwinkle populations, because the ultimate objective is not to provide sufficient protection for *Littorina* populations but for the entire coastal ecosystem.



## 5 Evaluation of the results

The results of the literature study were rated as satisfactory to derive biological TBT assessment criteria although it seems desirable to investigate in future projects the potential of TPT and other organotin compounds to induce intersex and to establish a broader data bases for the aqueous TBT concentration effect relationship (cf. chapter 3). It has been shown in section 4 that the ISI is the more robust and ecologically relevant parameter for the assessment of intersex intensities in periwinkle populations. Additionally, the ISI allows a better differentiation of TBT effects on the reproductive capability in *Littorina* populations.

One of the major problems of ISI determinations is the correct assessment of intersex intensities in only slightly TBT exposed populations. Therefore, experience of the analysts is critical for the correct assessment of coastal TBT contamination by means of ISI determinations. During the last BEQUALM and QUASIMEME training workshops on intersex and imposex measurements, which were held at the Marine Laboratory in Aberdeen, it has been shown that not sufficiently experienced analysts had difficulties to distinguish especially the intersex stages 0 and 1 so that a wrong determination of a normal female without intersex (stage 0) as a stage 1 specimen resulted already in elevated ISI values. The JAMP guideline requires a sample size of 40 specimens. Even in highly polluted areas, the sex ratio in *Littorina littorea* samples is balanced so that an average of 20 females can be expected in a normal sample. The wrong determination of a single female as a stage 1 individual in a sample unaffected from intersex results in an ISI value of 0.05. Consequently, it has to be strongly recommended that all laboratories performing intersex or imposex determination should participate in regular quality assurance and control programmes, including training workshops, such as offered by QUASIMEME.

Due to the broad available data base for intersex in periwinkles, based on measured ISI values in 318 samples (cf. Fig. 4), it can be shown that:

1. no sterilised periwinkles have been found so far in populations with an  $ISI \leq 0.10$ .
2. sterilisation in populations of *Littorina littorea* may occur at ISI values  $> 0.10$  and aqueous TBT concentrations as low as 2 ng as Sn/l, although only five examples of samples with sterilised females exhibiting an ISI range from 0.10 to 0.30 have been found in published reports so far (cf. Figs. 1a, 2a).
3. at ISI values  $> 0.30$  (equivalent to approximately 6 ng TBT as Sn/l) the occurrence of sterilised females in the samples may gradually increase and at ISI values  $> 0.70$  (equivalent to approximately 12 ng TBT as Sn/l) sterile females are generally present in all samples (cf. Figs. 1a, 2a).

4. virtually all *Littorina* females cease breeding at ISI values  $> 2.50$  (equivalent to approximately 23 ng TBT as Sn/l; cf. Fig. 1a, 2a).
5. *Littorina* is less sensitive to TBT compared with dog whelks, which are already sterilised at ambient TBT concentrations  $> 2.0$  ng as Sn/l with a complete loss of reproductive capability at  $> 10$  ng as Sn/l. The interspecific comparison of *Littorina* and *Nucella* shows that normally no sterilised dog whelk females occur in areas with ISI values  $< 0.30$  in periwinkles. In the ISI range between 0.30 and 0.50 the percentage of sterilised dog whelk females living sympatrically is gradually increasing and attains 100% indicating a complete cessation of breeding (cf. Fig. 3).
6. due to the above mentioned difficulties in the correct identification of intersex stage 1 females for unexperienced analysts, it is problematic to consider a differentiation of the TBT exposure level in periwinkle populations in the ISI range from 0 to 0.30.

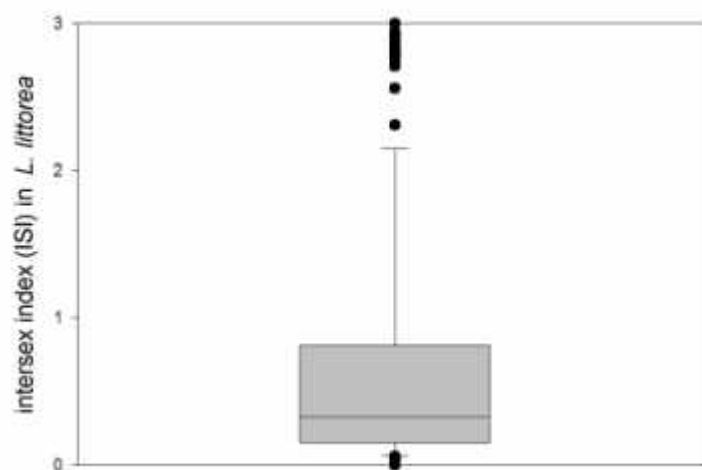


Fig. 4. Box-Whisker-Plot of all 318 considered ISI values in *Littorina littorea*. Dots represent extreme values, the error bar the range between the first (0.06) and ninth percentile (2.15), the grey box the range between the first (0.15) and third quartile (0.81), and the horizontal line the median value (0.325). Mean value = 0.690.

Table 2 summarises these findings and gives an indication of biological TBT effects on the coastal biocenosis at given ISI values in *Littorina littorea* populations.

Tab. 2. Summary of biological TBT effects in coastal ecosystems, which are likely at given intersex intensities, measured as intersex indices (ISI) in populations of *Littorina littorea*.

| ISI value     | Biological TBT effects   | Aqueous TBT concentration (ng as Sn/l) |
|---------------|--|--|
| < 0.10        | no adverse effects on the individual and population level  | < 2.0                                  |
| 0.10 – < 0.30 | single sterile snails may occur in populations but no adverse effects on the population level  | ≤ 2.0                                  |
| 0.30 – < 0.50 | up to 100% sterility in sympatrically living <i>Nucella</i> populations but no or neglectable effects on <i>Littorina</i> populations  | > 2.0 – 10                             |
| 0.50 – < 0.70 | <i>Nucella</i> populations are unable to reproduce; percentage of <i>Littorina</i> populations with sterile females increases but still only neglectable effects on periwinkle populations           | > 10 – 12                              |
| 0.70 – < 1.20 | <i>Nucella</i> populations are unable to reproduce; sterile females in all <i>Littorina</i> populations with an incidence of up to 30% so that a negative impact on periwinkle populations is likely | > 12 – 15                              |
| 1.20 – 2.50   | <i>Nucella</i> populations expired; incidence of sterile females in <i>Littorina</i> populations 30 - 80% resulting in poor reproductive success   | > 15 – 23                              |
| > 2.50        | <i>Nucella</i> populations expired; complete cessation of breeding in <i>Littorina</i> populations   | > 23                                   |

Annex V, table 1.2 of the EU WFD distinguishes five ecological status classes:

**High status (ecological status class I):**

There are no, or only very minor, anthropogenic alterations to the values of the physico-chemical and hydromorphological quality elements for the surface water body type from those normally associated with that type under undisturbed conditions.

The values of the biological quality elements for the surface water body reflect those normally associated with that type under undisturbed conditions, and show no, or only very minor, evidence of distortion.

These are the type-specific conditions and communities.

**Good status (ecological status class II):**

The values of the biological quality elements for the surface water body type show low levels of distortion resulting from human activity, but deviate only slightly from those normally associated with the surface water body type under undisturbed conditions.

**Moderate status (ecological status class III):**

The values of the biological quality elements for the surface water body type deviate moderately from those normally associated with the surface water body type under undisturbed conditions. The values show moderate signs of distortion resulting from human activity and are significantly more disturbed than under conditions of good status.

**Poor status (ecological status class IV):**

Waters showing evidence of major alterations to the values of the biological quality elements for the surface water body type and in

which the relevant biological communities deviate substantially from those normally associated with the surface water body type under undisturbed conditions, shall be classified as poor.

**Bad status (ecological status class V):**

Waters showing evidence of severe alterations to the values of the biological quality elements for the surface water body type and in which large portions of the relevant biological communities normally associated with the surface water body type under undisturbed conditions are absent, shall be classified as bad.

A central question of interest and crucial for the development of assessment criteria is the level of biological integration at which TBT effects are measurable. It is widely accepted in ecotoxicology that not the individual but the population level is the protection target, establishing a major difference to human toxicology (e.g. Walker et al., 1996; Fent, 1998). From a biological point of view it can therefore be argued that unless an effect has consequences at the population level it is insignificant. An alternative standpoint is that preventative action should be taken when effects are detected in individual animals. These different views are also expressed by the comments on the draft assessment criteria for TBT-specific biological effect in *Nucella lapillus*, presented by Denmark at the ASMO meeting in Ostend, 26–30 March 2001 (document ASMO 01/5/15-E(L)). The proposed assessment criteria for imposex in dog whelks, published in Harding et al. (1999), reflect the above mentioned standpoint that unless an effect has consequences at the population level it is not indicating any significant distortion from the normal situation and allows therefore not an assignment to the ecological status classes III or higher (cf. below). This assumption is also based on the fact that no other reported biological TBT effect rivals the imposex phenomenon in prosobranch, speaking in terms of sensitivity (e.g. Bryan & Gibbs, 1991). The Danish comments on the assessment criteria for imposex in *Nucella* at the ASMO meeting refer strictly to a specimen-based evaluation of biological effects of pollutant exposure, whereas the EU Water Framework Directive (WFD) refers to community responses (cf. below mentioned definitions of ecological status classes). Therefore, this report will also consider only population relevant biological effects of TBT for the development of assessment criteria for intersex in *Littorina littorea* as it has been done for imposex in *Nucella lapillus* by Harding et al. (1999), but it will take into account that biological TBT effects occur at higher ambient concentrations of this organotin compound in periwinkles compared to other imposex-affected prosobranch species.

Biological TBT assessment criteria according to the EU WFD for intersex in *Littorina littorea* have already been proposed by Wappelhorst et al. (2000) and Korhammer et al. (2001). These criteria are presented in table 3.

Tab. 3. Biological TBT assessment criteria for intersex in *Littorina littorea* according to the EU Water Framework Directive as proposed by Wappelhorst et al. (2000) and Korhammer et al. (2001).

| Ecological status class | ISI value     | Description  |
|-------------------------|---------------|--|
| I<br>(high)             | < 0.10        | The effects on <i>Littorina littorea</i> specimens are low and reflect an undisturbed condition with little, if any, signs of anthropogenic distortion. Because TBT is a xenobiotic of exclusively anthropogenic origin, ISI values should be generally below 0.1 in this status class.  |
| II<br>(good)            | 0.10 - < 0.30 | The effects on <i>Littorina littorea</i> indicate low levels of anthropogenic distortion, but deviate only slightly from those under undisturbed conditions. Sterile females may occur as isolated cases in periwinkle populations, but no restrictions of the reproductive capability on the population level are assessable.   |
| III<br>(moderate)       | 0.30 - < 0.70 | The effects on <i>Littorina littorea</i> indicate moderate and significant levels of anthropogenic distortion. Reproduction in <i>Littorina</i> populations with only little signs of impairment, but reproduction is significantly affected in more sensitive taxa of the coastal ecosystem such as <i>Nucella lapillus</i> or other imposex-affected prosobranchs                            |
| IV<br>(poor)            | 0.70 - < 1.20 | The effects on <i>Littorina littorea</i> indicate major alterations and substantial deviations of relevant biological communities from those under undisturbed conditions. Even reproduction of periwinkle populations is negatively affected with an incidence of up to 30% sterile females.  |
| V<br>(bad)              | $\geq 1.20$   | The effects on <i>Littorina littorea</i> indicate severe alterations and the absence of large portions of relevant biological communities, which are normally associated with undisturbed conditions. In most cases, more than 50% of the females in periwinkle populations are sterile. Imposex affected species such as <i>Nucella lapillus</i> and <i>Ocenebrina aciculata</i> have expired |

The main advantage of the assessment scheme proposed by Wappelhorst et al. (2000) and Korhammer et al. (2001) is that it is based on an ecological and ecotoxicological evaluation of the effects of intersex development on the population level and that it considers also the higher sensitivity of imposex affected species with special emphasis on *Nucella lapillus*. The literature survey in the current study and the resulting broader data base requires no changes of the original scheme with regard to the ISI ranges if ecological and ecotoxicological criteria are considered.

## 6 Recommendations for biological assessment criteria

Based on the evaluation of TBT effects on intersex development and reproductive capability in the periwinkle *Littorina littorea* and also considering the effects of TBT in sympatrically living populations of imposex affected prosobranch snails (with special emphasis on *Nucella lapillus*), it is recommended to use the biological TBT assessment criteria as already outlined by Wappelhorst et al. (2000) and Korhammer et al. (2001), but with a common ISI range for the ecological status classes I and II. This alteration considers the limited practicability to differentiate between two status classes in the narrow ISI range of 0 to 0.30 and also that periwinkles are less sensitive to TBT in comparison to other imposex-affected prosobranch species, which seem to be better suited for the assessment of biological TBT effects at lower environmental concentrations. On the other hand, the assessment of intersex intensities in periwinkle populations has an advantage compared to imposex in *Nucella lapillus* in highly contaminated areas, because dog whelk populations are likely to become extinct in such regions. The recommended assessment criteria are summarised in table 4, amended with a prediction of VDSI values and biological TBT effects in sympatrically living populations of the dog whelk, *Nucella lapillus*.

These criteria are not only in line but rather complement those proposed for the imposex response in dog whelks by Harding et al. (1999), based on a survey of imposex in North Sea *Nucella* population in 1992 (Tab. 5).

Tab. 4. Recommended biological TBT assessment criteria for intersex in *Littorina littorea* according to the EU Water Framework Directive with a prediction of VDSI values and biological TBT effects in sympatrically living populations of the dog whelk, *Nucella lapillus*.

| Ecological status class | ISI value     | Description  |
|-------------------------|---------------|--|
| I – II<br>(high – good) | < 0.30        | The effects on <i>Littorina littorea</i> specimens are low and reflect a condition with little signs or low levels of anthropogenic distortion. Sterile females may occur as isolated cases in periwinkle populations, but no restrictions of the reproductive capability on the population level are assessable.<br>No adverse effects of TBT in sympatrically living dog whelks at the population level. VDSI values in <i>Nucella lapillus</i> range from 0 – < 4.00. |
| III<br>(moderate)       | 0.30 - < 0.70 | The effects on <i>Littorina littorea</i> indicate moderate and significant levels of anthropogenic distortion. Reproduction in <i>Littorina</i> populations with only little signs of impairment, but reproduction is significantly affected in more sensitive taxa of the coastal ecosystem such as <i>Nucella lapillus</i> (VDSI range from 4.00 - < 5.00; percentage of up to 100% sterile females) or other imposex-affected prosobranchs                            |
| IV<br>(poor)            | 0.70 - < 1.20 | The effects on <i>Littorina littorea</i> indicate major alterations and substantial deviations of relevant biological communities from those under undisturbed conditions. Even reproduction of periwinkle populations is negatively affected with an incidence of up to 30% sterile females.<br><i>Nucella</i> populations are unable to reproduce (100% sterile females) with VDSI values $\geq 5.00$  |
| V<br>(bad)              | $\geq 1.20$   | The effects on <i>Littorina littorea</i> indicate severe alterations and the absence of large portions of relevant biological communities, which are normally associated with undisturbed conditions. In most cases, more than 50% of the females in periwinkle populations are sterile.<br>Imposex affected species such as <i>Nucella lapillus</i> and <i>Ocenebrina aciculata</i> have expired  |

Tab. 5. Comparison of recommended biological TBT assessment criteria for intersex in *Littorina littorea* and predicted VDSI values in sympatrically living populations of the dog whelk, *Nucella lapillus* with those assessment criteria proposed by Harding et al. (1999) for imposex in *N. lapillus*.

| Species                                   | Parameter | Ecological status classes |    |               |               |             |
|---|-----------|---------------------------|----|---------------|---------------|-------------|
|   |           | I                         | II | III           | IV            | V           |
| <i>Littorina littorea</i><br>(this study) | ISI       | < 0.30                    |    | 0.30 - < 0.70 | 0.70 - < 1.20 | $\geq 1.20$ |
| <i>Nucella lapillus</i><br>(predicted)    | VDSI      | < 4.00                    |    | 4.00 - < 5.00 | $\geq 5.00$   | Expired     |

| Species  | Parameter | Categories |               |               |             |
|--|-----------|------------|---------------|---------------|-------------|
|  |           | A          | B             | C             | D           |
| <i>Nucella lapillus</i><br>(Harding et al. 1992) | VDSI      | < 2.00     | 2.00 - < 4.00 | 4.00 - < 5.00 | $\geq 5.00$ |

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