Biological effects of contaminants:
Use of intersex in the periwinkle (*Littorina littorea*) as a biomarker of tributyltin pollution

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ICES Techniques in Marine Environmental Sciences

Biological effects of contaminants: Use of intersex in the periwinkle (Littorina littorea) as a biomarker of tributyltin pollution


Abstract

The method described here is for detecting contamination of the marine environment by the biocide tributyltin (TBT) using the prosobranch snail Littorina littorea (L., 1758) as a bioindicator. This species is widely distributed in European coastal waters and on the east coast of North America. The method has been tested nationally in Germany as well as in international laboratory performance studies under the Joint Assessment and Monitoring Programme (JAMP) of the OSPAR Commission, as organized by QUASIMEME. Exposure of female L. littorea to TBT induces a masculinization, which has been termed “intersex”. The indices that have been employed to measure intersex in L. littorea are described, together with a brief account of the biology of this organism.

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Keywords: tributyltin, TBT, bioindicator, periwinkle, Littorina littorea, intersex, biological effects of contaminants.
INTRODUCTION

Tributyltin (TBT) compounds have 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). They are primarily used as biocides in antifouling paints that are applied to ship hulls, and harbour and offshore installations to prevent the attachment of sedentary organisms, such as algae, polychaetes, mussels, and barnacles. Furthermore, TBT is used as a biocide in wood preservatives and various other formulations and has been reported to be an important contaminant of mono- and dibutyltin additives used in many consumer products. The high efficacy of TBT-based antifoulants led to wide usage in the 1970s on pleasure boats and deep sea-going vessels with market shares up to 80%, resulting in aqueous concentrations in the $\mu g \, l^{-1}$ range near harbours and marinas.

At the same time, impacts of TBT on non-target organisms became evident. Today, this organotin compound is known to produce a variety of malformations in marine animals, with molluscs being one of the most TBT-sensitive groups of invertebrates (for a review, see Bryan and Gibbs, 1991; Matthiessen and Gibbs, 1998). In particular, the shell malformations observed in cultivated oysters \textit{(Crassostrea gigas)} triggered legislative restrictions for antifouling paints to reduce TBT contamination in coastal waters. France was the first country to draw up such regulations and banned the use of TBT antifoulants on small boats (length <25 m) in 1982. Since 1987, other countries have adopted similar legislation, yet TBT levels in coastal waters continue to be elevated and have even increased in some regions. Consequently, in autumn 2001 the International Maritime Organization (IMO) decided to ban the application of TBT-based paints on all vessels as of January 2003, and the presence of leachable TBT-based paints on ship hulls and external surfaces as of January 2008 (IMO, 2001). The antifouling convention of IMO will enter into force twelve months after the date on which 25 states, the combined merchant fleets of which constitute not less than 25% of the gross tonnage of the world’s merchant shipping, have ratified it.

One of the first described impacts of TBT, although not attributed to it at the time, was the masculinization of female prosobranch snails, termed “imposex” (Blaber, 1970; Smith, 1971). About ten years later, Féral (1980) and Smith (1981) were the first to provide evidence that exposure to TBT was the cause of imposex. Yet it was not until the late 1980s, when suitable methods for organotin analysis were available, that the high sensitivity of this pathomorphological response was demonstrated. Since then, imposex in prosobranchs has been successfully used as a biomarker for monitoring environmental TBT pollution and the potential amelioration of the exposure situation following legislative controls. The majority of imposex studies have been focused on the dogwhelk \textit{Nucella lapillus}. This species is readily available on many European coasts and on the east coast of North America; it has a well-characterized ecology and reproductive biology, and has also proved to be one of the most sensitive species to TBT (Bryan et al., 1987; Gibbs et al., 1987; Oehlmann et al., 1991; Harding et al., 1999). Other prosobranch species have also occasionally been employed in European imposex surveys (e.g., Stroben et al., 1992; Oehlmann et al., 1993; Ten Hallers-Tjabbes et al., 1994).

Despite its generally broad distribution, the dogwhelk does not occur in some European coastal regions such as in the entire Baltic Sea and the southern part of the North Sea, mostly owing to unsuitable habitats. In addition, \textit{Nucella} populations can be eliminated near TBT point sources, such as in harbours and marinas, due to the high level of TBT pollution. The need for a bioindicator for areas where dogwhelks and other neogastropods are absent led to investigations of possible TBT effects on the periwinkle \textit{Littorina littorea}. Bauer et al. (1995, 1997) were the first to describe intersex in this mesogastropod, a phenomenon characterized by a transformation of the pallial oviduct towards a male morphology with a final supplanting of...
female organs by the corresponding male formations. Later studies have shown that *L. littorea* is well suited for TBT effect monitoring because: a) it is tolerant of high TBT levels, b) it recruits from the plankton, and c) it can occur in areas where dogwhelks have been eliminated. The species is very common, and sufficient numbers are available for sampling, even in areas with a relatively high level of contamination.

Thus, intersex in *Littorina littorea* and imposex in the dogwhelk *Nucella lapillus* were selected for use in biological TBT effect monitoring surveys within the Joint Assessment and Monitoring Programme (JAMP) of the OSPAR Commission. Both effects have been rated as perhaps the best-documented examples of endocrine disruption in invertebrates (Matthiessen and Gibbs, 1998).

This document describes the technical aspects of intersex determination in *Littorina littorea*, including indices to determine the degree of masculinization in periwinkle populations. Differences in TBT sensitivity between *L. littorea* and *Nucella lapillus* are addressed. Since intersex in periwinkles and imposex in dogwhelks are commonly investigated within the OSPAR JAMP, this document supplements the TIMES publication on imposex in *N. lapillus* (Gibbs, 1999) and, thus, is structured in a parallel way. The focus is on the most important aspects for measurement, interpretation, and evaluation of intersex as a marker of biological effects of TBT and citations have therefore been kept to a minimum. More detailed information can be found in papers by Bauer *et al.* (1995, 1997), Davies *et al.* (1999), and Oehlmann *et al.* (1998a, 1998b).

All tributyltin concentrations are given as Sn (TBT-Sn or TBT as Sn); they can be converted to TBT by multiplying by 2.44.

## 2 THE PERIWINKLE (*Littorina littorea*)

### 2.1 General Biology

Details of the biology of *Littorina littorea* are described in Linke (1933), Moore (1937, 1940), Fretter and Graham (1980), and Reid (1996). *L. littorea* is a shallow-water species that lives mainly on rocky shores but can also be found on sandy and muddy coastlines (such as in the Wadden Sea), where stones, gravel, shells, wood, mussel beds, or other sediment materials provide refuges of firm attachment. It is usually abundant on sheltered shores and generally absent from the most exposed areas. Normally, densities do not exceed 200 per m² in Europe, although under exceptional conditions a maximum of 17,500 per m² has been reported. In New England, densities appear to be higher, even on average shores, often exceeding 800 per m².

The majority of ecological studies of periwinkles have been carried out on rocky shores, where the species is a characteristic inhabitant of levels between the high-water mark of neap tides and the extreme low-water mark of spring tides. The species is found in the zones of *Fucus vesiculosus* and *Ascophyllum nodosum*, of *Fucus serratus*, and extends into the Laminaria zone. Though the bulk of the population occurs intertidally, records of occurrence below the immediate sublittoral have often been made in the colder and more northerly parts of its range, with maximum depths of 35 m in the Gulf of St. Lawrence and 60 m in northern Britain. The geographical distribution ranges from southern Portugal to southern Spitzbergen in the Northeastern Atlantic and from New Jersey to Greenland in the Northwestern Atlantic. Periwinkles feed largely on epilithic algae and vegetable detritus, although they may occasionally feed on dead animal matter as a scavenger.
The sexes are separate. Females reach maturity at shell heights of 10–15 mm and at an age of 12–18 months; they produce about 500 planktonic egg capsules, each containing 1–5 eggs (Figure 1). In most regions, periwinkles breed from December to May, but longer reproduction phases have been reported especially at the southern limit of their distribution. After 5–6 days, the animals hatch as free-swimming veliger larvae drifting with the plankton. Metamorphosis occurs after 4–7 weeks. Although the larvae exhibit a bilobic velum and spend a considerable time in the plankton, they are typical “inshore plankton” and are not considered as true long-distance veligers. Adult *Littorina littorea* can live for more than nine years in captivity, while in the field, individuals have been observed to survive at least five years.

The shell height of mature periwinkles ranges from 11 mm to 53 mm. This considerable variation is due to the habitat, particularly to the salinity of the ambient water. The largest specimens are found in the north of the range, at a salinity of 35 PSU, while the smallest individuals occur in brackish estuaries and especially in the southwestern part of the Baltic. *Littorina* specimens tend to develop thicker shells in areas where the crab *Carcinus maenas* is the main predator.

### 2.2 Reproductive Features

#### 2.2.1 Genital system of *Littorina littorea*

The anatomy and histology of the normal female and male genital tract of *Littorina littorea* are described in detail by Linke (1933), Fretter and Graham (1962), Fretter (1980), and Reid (1989). In both sexes a gonadial, renal, and pallial section of the genital tract can be distinguished. The testis and ovary are large and diffuse organs, lying in the upper parts of the visceral complex or hump, branching between the tubules of the midgut gland (Figure 2). The most proximal parts of the male and female genital tract are the gonadial sections of the vas deferens and oviduct, which are formed by gonad tissue. These run parallel to the posterior aorta at the columellar side of the visceral complex and continue in the rather short renal section, which is the remnant of the right kidney of less evolved prosobranch snails (Archaeogastropoda).
Figure 2. *Littorina littorea* female removed from shell and seen from the right side (modified from Fretter and Graham, 1962).

The most complex part of the reproductive tract of *Littorina littorea* is the pallial (= mantle) section of males and females, which originates ontogenetically from an infolding of the mantle epithelium. The pallial section of males is an open sperm groove over its entire length (Figure 3A). The most proximal parts of the walls of the groove are tall and are characterized by the development of folds of glandular tissue, forming a well-developed prostate gland. From the prostate, the sperm groove runs forward on the floor of the mantle cavity to the base of the penis behind the right ocular tentacle. The copulatory organ is conical, flattened laterally, and carries the sperm groove as the so-called penis duct to its summit along the dorsal edge. Numerous mamilliform penial glands can be found on the ventral border of the penis. Their secretions hold the penis in position during copulation. Over most of the geographical range, male periwinkles shed their penis after the breeding season. Therefore, from June to September, most males within a population have no copulatory organ except the penis base, which resembles the ovipositor of females in shape and structure (see below). A new penis is then built up in late summer or autumn.

The pallial section of females is highly developed in order to store sperm, to provide extraembryonic nourishment for the embryos within the capsule, and to produce the planktonic egg capsules. It consists of a receptaculum seminis, and an albumen gland, a capsule gland, and a jelly gland, completed by the bursa copulatrix in ventro-lateral position, and the vagina (Figure 3B). The compact part of the capsule gland (ccg in Figure 3B) provides a characteristic feature of the pallial oviduct section in periwinkles, because it is visible as a white-coloured “W” when seen from the right (Figure 4B). In adult females, the pallial oviduct is a closed tube originating from an infolding of the mantle epithelium with a consecutive fusion of its flaps. The ovoid-shaped vaginal opening is the only aperture towards the mantle cavity. Distally from the vaginal opening, an egg channel extends over the floor of the mantle cavity and runs down the right side towards the foot. It ends in the so-called ovipositor. The ovipositor is a muscular and glandular organ with the function of launching the egg capsules on their pelagic life phase. Its topographical position, and histological and ultrastructural properties, are identical with the
base of the male penis that remains after the periodic shedding of the copulatory organ during the sexual repose phase.

**Figure 3.** *Littorina littorea*. Diagrams of the pallial gonoduct sections of a normal male (A) and a normal female (B) seen from the right side (from Oehlmann et al., 1998b).

Abbreviations: ag = albumen gland; bc = bursa copulatrix; ccg = compact part of capsule gland; ct = connective tissue; ec = egg channel; jg = jelly gland; od = oviduct; op = ovipositor; p = penis; pd = penis duct; pg = penis glands; pr = prostate; pre = prostate epithelium; rs = receptaculum seminis; sg = sperm groove; tcg = translucent part of capsule gland; vo = vaginal opening; vs = vesicula seminalis.

### 2.2.2 Routine determination of sex

The sexes cannot be distinguished on the basis of shell characters. Although females tend to have larger shells and a larger body weight, these differences are not reliable. Since males are able to shed their penis, it is also not possible to determine sex based on the presence or absence of the male copulatory organ, without first sacrificing the organisms.

For routine determination of sex and the subsequent examination of intersex, the soft parts of the snails must be extracted from the shell. *Littorina littorea* specimens are sedated by exposure to 7% MgCl₂ in distilled water (for Atlantic and North Sea samples; other animals may require a lower concentration depending on the salinity at sampling sites). Maximum relaxation is
achieved after approximately two hours and the shell is then cracked open with a bench vice. The columellar muscle is detached by gently teasing or cutting its attachment area to the shell. Finally, the snails are laid on their left side and covered by the narcotizing solution so that the right side is visible, as illustrated in Figure 4A–C. The most convenient and reliable features to distinguish between males and females are the texture and colour of the gonad during the breeding season and the structure of the gonaduct at the collumellar side of the visceral hump at other times of the year. The parameters used in determining sex are summarized as a decision tree in Figure 5.

**Figure 4.** *Littorina littorea.* Photographs of a normal male (A) and a normal female (B) during the breeding season, seen from the right side. (C) provides the same view of a male during the sexual repose phase. Abbreviations: od = oviduct; ov = ovary; te = testis; vs = vesicula seminalis.
The testis of males is yellow and characterized by a smooth texture during the period when most periwinkle populations breed within the species’ range, between December and May. Additionally, the heavily coiled vesicula seminalis, which acts as a storage organ for sperm, is swollen and pearly white in colour (Figure 4A). At the same time, the ovary of females is light red or pale pink in colour and has a granular appearance due to the presence of the large yolk-rich oocytes, which can be observed through the translucent epithelium of the visceral hump. The posterior part of the oviduct, the so-called gonadal and renal section, is, in contrast to the vesicula seminalis in males, not coiled but is a straight channel, normally filled with red- or pink-coloured oocytes (Figure 4B).

From June to November, during the species’ sexual repose phase, the gonad in both sexes is normally brownish or has another colour (light or dark grey or creamy yellow). Since most females no longer have ripe oocytes within the ovary, this organ loses its granular texture, and therefore sexes cannot be identified on the basis of the gonad’s structure and colour. Although the vesicula seminalis of males is no longer sperm-filled and thus not white-coloured, this organ is still heavily coiled (Figure 4C), whereas the corresponding gonoduct section of females, the gonadal and renal oviduct, is again a straight structure. Even after the breeding season, single oocytes remain in this oviduct section, facilitating its identification.

Figure 5. Decision tree for routine determination of sex in Littorina littorea.

Ideally, a sample of periwinkles could be expected to be composed of equal numbers of the two sexes, but in practice there is commonly a weak predominance of females (see also Annex 1). This shift of the sex ratio in favour of females is generally not statistically significant and seems to be a general characteristic of prosobranch populations. In contrast to the dogwhelk, this trend is not reversed, even in Littorina populations exposed to high ambient TBT concentrations, because the development of intersex does not cause an increased mortality of females, as is the case for imposex in Nucella lapillus and other muricid gastropods.

3 INTERSEX RESPONSE

3.1 Definition, Development, and Effect

A common feature of intersex and imposex is the gradual TBT-induced increase of virilization that finally leads to female sterility. In imposex-affected species, such as Nucella lapillus, the
entire female genital system is conserved but superimposed (thus the term “imposex”) by male organs such as a penis and/or vas deferens. However, in *Littorina littorea* the female pallial organs are modified towards a male morphological structure in lower intersex stages (1, 2) and then supplanted by the corresponding male formation, a prostate gland (stages 3 and 4). Both phenomena, intersex and imposex, can be described by using evolutive schemes which were first introduced by Bauer et al. (1995) for intersex, with four different stages (Figure 6), and by Gibbs et al. (1987) for imposex development in *Nucella lapillus*, with six stages. There is now considerable evidence that both phenomena result from a hormonal imbalance as a consequence of TBT exposure and represent examples of endocrine disruption in invertebrates (Matthiessen and Gibbs, 1998).

**Figure 6.** *Littorina littorea* (female). Intersex development scheme with four different stages. Dorsal views with opened mantle cavity (left) and lateral views of the pallial section of the genital tract (right) (from Oehlmann et al., 1998a).

Abbreviations: a = anus; ag = albumen gland; bc = bursa copulatrix; cg = capsule gland; jg = jelly gland; obc = open bursa copulatrix; opo = open pallial oviduct; p = penis; po = pallial oviduct; pr = prostate; rs = receptaculum seminis; sg = sperm groove; vo = vaginal opening.
The intersex phenomenon of *Littorina littorea* is a gradual transformation of the female pallial tract. Although the alterations in the genital system of periwinkles associated with the development of intersex are a continuum, depending on the degree of TBT exposure in still sexually immature specimens (Bauer *et al.*, 1997), its progress can be used to score the intensity of its expression. Four main stages, based primarily on the structure of the pallial oviduct section, are readily identifiable in *L. littorea* using a stereomicroscope.

**Stage 0** Normal female without any intersex characteristics (Figure 7).

The entire pallial oviduct is a ventrally closed tube with the vulva representing the only aperture to the mantle cavity. The vaginal opening is symmetrical and ovoid-shaped without any signs of a slit-like prolongation in the posterior direction. The actual size of the vaginal opening may vary considerably, especially during the breeding season.

The identification of this stage requires opening the mantle cavity by a longitudinal cut through the hypobranchial gland in the mantle roof (between the gill and the rectum).

**Figure 7.** *Littorina littorea*. Scanning electron micrographs of a normal female (intersex stage 0). (A) View into the anterior part of the mantle cavity. (B) Detail of (A) with the symmetrical, ovoid-shaped vaginal opening (from Oehlmann *et al.*, 1998b).

Abbreviations: a = anus; er = egg channel; ov = ovipositor; r = rectum; vo = vaginal opening.

**Stage 1** The bursa copulatrix is split ventrally exposing its internal lobes (Figure 8).

TBT has inhibited the complete fusion of the free edges of the pallial oviduct section during ontogenetic development in the most anterior part. Consequently, the vaginal opening is no longer symmetrical, and exhibits a slit-like aperture in the posterior direction so that the bursa copulatrix is split ventrally. Figure 8 provides photographs of two quite early examples of stage 1 females. In more advanced cases, the entire bursa may be split ventrally (to up to 1/3 of the length of the pallial oviduct section). Sperm, which are transferred by the male into the bursa during copulation, may leak into the mantle cavity thus reducing the reproductive success of the female.

The identification of this stage requires opening the mantle cavity.

**Stage 2** The pallial oviduct (i.e., bursa copulatrix and jelly gland) is split ventrally (Figure 9).

TBT has completely inhibited the fusion of the free edges of the pallial oviduct section during ontogenetic development. Consequently, not only the bursa
copulatrix, but also at least the jelly gland, is split ventrally (more than 1/3 of the length of the pallial oviduct section). This structure of the pallial oviduct is a male characteristic because the prostate gland of males is also an open groove (Figure 3A). Stage 2 represents the first functionally sterilized stage of intersex development because oocytes and capsular material produced by the female gland complex in the pallial oviduct section leak into the mantle cavity so that the female can no longer produce intact egg capsules.

The identification of this stage requires opening the mantle cavity.

**Figure 8.** *Littorina littorea*. Intersex stage 1 female characteristics shown by scanning electron micrograph (A) and fresh specimen (B) showing the slit-like aperture of the bursa copulatrix to the mantle cavity (from Oehlmann et al., 1998b).

Abbreviations: a = anus; obc = open bursa copulatrix; r = rectum; vo = vaginal opening.

**Figure 9.** *Littorina littorea*. Intersex stage 2 female characteristics shown by scanning electron micrograph (A) and fresh specimen (B) showing the ventrally open pallial oviduct section (from Oehlmann et al., 1998b).

Abbreviations: a = anus; er = egg channel; obc = open bursa copulatrix; okd = open pallial oviduct.
Stage 3  The anterior part of the pallial oviduct (capsule and jelly glands with bursa copulatrix and vagina) is partially or totally supplanted by a prostate gland (Figure 10). In early examples of stage 3, short and isolated pieces of prostate tissue can be found within the female glands, typically at the dorsal edge of the jelly and capsule glands. In more advanced cases, first the jelly and capsule glands and finally also the albumen gland and the receptaculum seminis, are supplanted completely by prostate tissue, as indicated in Figure 10. Prostate tissue can easily be identified by the characteristic vertical stripes at the dorsal edge of the organ. The intersex stage 3 is attained as soon as a female has developed prostate tissue, irrespective of the extension of this male formation. In some stage 3 specimens, the free edges of the open prostate gland fuse to form a closed tube in order to establish a more female anatomical structure. The line of fusion of the prostate edges is detectable by its white coloration, after opening the mantle cavity. Stage 3 females are unable to reproduce because the glands responsible for the formation of the planktonic egg capsule are at least partially missing. This stage can be identified from external inspection without opening of the mantle cavity.

Stage 4  In addition to the characteristics of stage 3, a penis with an open sperm groove is developed. This stage provides some phenotypic similarities with the imposex response in other prosobranch species, such as the dogwhelk *Nucella lapillus*. It is worth mentioning that, historically, it is by far the least frequent intersex stage in field populations, with only two examples identified from more than 15,000 females analysed from European coasts.

Figure 10. *Littorina littorea*. External view of an intersex stage 3 female. The pallial oviduct is supplanted by the prostate gland. The longitudinal line along the prostate indicates how to measure the length of the organ.

Abbreviations: *od* = gonadal and renal oviduct section; *ov* = ovary; *pr* = prostate gland.

A common characteristic of intersex and imposex is that both are irreversible pathomorphological developments in prosobranch snails. Even if affected dogwhelk females are transferred to a TBT-free environment for long periods, imposex does not revert to a lower stage or return to the normal imposex-free condition. This has been noted in other species as well as in laboratory experiments with *Littorina littorea* (Bauer *et al.*, 1997).
However, there are a number of differences between these two phenomena that have important implications for the interpretation of data (see Section 4):

- The final result of intersex development is the sterilization of females. Yet, in contrast to the impairment of breeding observed in the advanced stages 5 and 6 of imposex, the reproductive performance of female *Littorina littorea* is already affected at the initial intersex stages, although at higher aqueous TBT concentrations. The ventrally open bursa copulatrix in intersex stage 1 inhibits successful copulation because sperm can be spilled into the mantle cavity. *L. littorea* is definitely sterilized in stages 2, 3, and 4, either because the oocytes and capsular material leak into the mantle cavity (stage 2), or the glands responsible for the formation of the planktonic egg capsule are at least partially missing.

- Though the ultimate effect of intersex and imposex development is sterilization of females, there are differences between the consequences of dogwhelk and periwinkle female sterility at the population and ecosystem levels. *Nucella lapillus* produces benthic egg capsules and the offspring develop within the capsule, hatch and crawl onto the shore without passing through a pelagic larval stage. Because of this absence of a planktonic dispersal stage, dogwhelk populations will die out if all females in a given population cease breeding. Additionally, sterilized dogwhelk females suffer from a higher mortality and populations at highly contaminated stations are thus characterized by a shift of the sex ratio in favour of males (Gibbs, 1999). In contrast to the imposex phenomenon in dogwhelks and other muricids, sterilization due to intersex development in *Littorina littorea* results in poor recruitment of juveniles, but does not necessarily lead to the elimination of the local population. Provided that aqueous TBT concentrations are not toxic to the developing larvae (Matthiessen *et al.*, 1995), planktonic egg capsules from other less affected populations can contribute to the survival of the species. Intersex development does not result in increased female mortality because the oviduct does not become blocked. For this reason, the sex ratio in periwinkles remains balanced even in highly contaminated areas.

- Dogwhelks and periwinkles show less sensitivity to TBT as they develop into adults. Yet the differences in TBT sensitivity between sexually mature and immature specimens are by far more distinct in *Littorina littorea*. It has been demonstrated that the intersex condition can only be induced by TBT in sexually immature periwinkles (Bauer *et al.*, 1997). Once the normal genital tract has been formed (intersex stage 0 female), exposure to TBT no longer results in the development of male features that are characteristic of intersex. In contrast to periwinkles, sexually mature dogwhelk females are still able to develop additional male organs, such as a penis and/or vas deferens, when exposed to TBT for the first time.

- Intersex in *Littorina littorea* is induced at higher aqueous TBT concentrations than imposex in *Nucella lapillus*, thus representing a slightly less sensitive biological marker of TBT exposure (Oehlmann *et al.*, 1998a).

### 3.2 Measures of Expression or Intensity

A number of measures can be used to express the degree or intensity of intersex development in a population and each index can be useful when applied to an appropriate situation. The intersex index (ISI) is ecologically relevant, but requires some experience and training in the identification of intersex stages. The ISI is a particularly useful measure in slightly to moderately contaminated areas (< 2 ng TBT-Sn l\(^{-1}\)), whereas the mean female prostate length (FPrL) is only applicable at sites suffering from high TBT exposure (> 15 ng TBT- Sn l\(^{-1}\)). The
percentage of sterile females gives a clear indication of any reduction in breeding activity at ambient TBT concentrations > 6 ng Sn 1\(^{-1}\).

### 3.2.1 Intersex Incidence (proportion of affected females)

In areas where only a fraction of the females within the populations exhibit intersex, the percentage of intersex-affected periwinkles can be used as a measure to determine the degree of the effect. This parameter becomes redundant at higher TBT concentrations since most, if not all, females typically develop intersex characteristics in this situation, resulting in an intersex incidence of > 80%. This makes it difficult to differentiate between the exposure levels in such areas on the basis of the intersex incidence.

### 3.2.2 Intersex Index (ISI)

For *Littorina littorea*, the intersex index (ISI) was first introduced by Bauer *et al.* (1995) as the mean value of intersex stages in a given sample:

\[
ISI = \frac{\sum IS}{n_f}
\]

where

- \(IS\) = intersex stage of individual female
- \(n_f\) = number of females in sample.

This index is the equivalent of the vas deferens sequence index (VDSI) in imposex-affected prosobranch snails. ISI determinations require a minimum training of the investigator. The principal procedure for the most appropriate identification of intersex stages in periwinkles is summarized in Figure 11.

**Figure 11.** Decision tree for identification of intersex stages in *Littorina littorea* and additional measurements to determine intersex expression or intensity in populations.
Training workshops have revealed that the discrimination between intersex stages 0 and 1 is difficult for inexperienced analysts (Davies et al., 1999). Nevertheless, the intersex index exhibits a number of advantages compared to the other parameters mentioned in this section and is used as the primary measure of intersex under the JAMP:

- ISI values are independent of the size of snails in the population (in contrast to FPrL values).
- ISI values are obtained over a broad spectrum of environmentally relevant aqueous TBT concentrations (between < 2–30 ng TBT- Sn l$^{-1}$, cf. Section 4).
- ISI values provide a good estimate of the reproductive capability of females in the population (Figure 12). The loss of female fecundity is by far the most important effect during intersex development and has the greatest ecological relevance.

**Figure 12. Littorina littorea.** Relationship between the incidence of sterile females and the intersex index in French and Irish populations with calculated correlations. $y = 34.7x - 8.09$, $n = 268$ samples from 172 stations, $r = 0.982$, p < 0.0005.

As demonstrated in Figure 12, the first sterile female periwinkles may be found in a given population when the ISI attains a value of 0.10 and they can be expected at ISI values > 0.30. At values > 0.70, at least some females in the population are sterile, while virtually all females will have ceased breeding at ISI values above 2.50.

### 3.2.3 Mean Female Prostate Length (FPrL)

In females, prostate tissue is easily identifiable. It is also relatively easy to measure the length of this organ in the pallial gonoduct section (as indicated in Figure 10):
\[ F_{PrL} = \sum \frac{PrL}{n_f} \]

where

- \( PrL \) = prostate length of individual female (mm)
- \( n_f \) = number of females in sample.

The FPrL is less sensitive than the ISI because females do not develop a prostate gland before they reach the intersex stage 3. The JAMP guidelines propose that the FPrL be used as a secondary index to differentiate *Littorina* populations in highly contaminated areas, where almost all samples have ISI values near 3.0.

### 3.2.4 Proportion of sterile females

As a measure of the degree of intersex, the percentage of sterile females within a sample is also insensitive. Female periwinkles are definitively sterilized when they attain the intersex stage 2, while sterile periwinkles are generally not found at aqueous TBT concentrations below 6 ng Sn \( l^{-1} \). Consequently, this measure of intersex expression or intensity is only useful in highly contaminated areas.

## 4 INTERPRETATION OF DATA

The relationship between aqueous concentrations of TBT, expressed as ng Sn \( l^{-1} \), and the degree of intersex, expressed as ISI, in *Littorina littorea* is presented in Figure 13. ISI values below 0.10, indicating the absence of sterilized females, are associated with TBT concentrations as low as 2 ng Sn \( l^{-1} \) in water. Sterile females begin to be observed at aqueous TBT concentrations of 6 ng Sn \( l^{-1} \), at a threshold ISI value of 0.30. At a TBT concentration of 23 ng Sn \( l^{-1} \) and an ISI of 2.50, the reproductive capability of periwinkle populations is virtually nil. These statistically derived data agree with Matthiessen *et al.* (1995). They reported poor recruitment of English *Littorina* populations at ambient TBT concentrations of 18.4 ng Sn \( l^{-1} \), while populations recovered when TBT concentrations decreased below 2.9 ng Sn \( l^{-1} \), following legislative restrictions.

It is evident that dogwhelks are far more sensitive to TBT than periwinkles if one compares the relationship between TBT concentration and ISI in *Littorina littorea* with the correlation between TBT concentration and imposex intensity in *Nucella lapillus* (see Oehlmann *et al.*, 1998a). The threshold concentration for the induction of imposex is 0.5 ng TBT-Sn \( l^{-1} \) in *Nucella* (Gibbs *et al.*, 1987), while the corresponding value for intersex in *Littorina* is 2.0 ng TBT-Sn \( l^{-1} \). Female dogwhelks are sterilized at ambient TBT concentrations > 2.0 ng Sn \( l^{-1} \), with a complete loss of their reproductive capability at > 10 ng Sn \( l^{-1} \), while the corresponding values are 6 ng Sn \( l^{-1} \) and 23 ng Sn \( l^{-1} \) for periwinkles.

The difference in TBT sensitivity between these species was confirmed in the field, when both prosobranchs were used in monitoring surveys to assess the degree of TBT contamination in a given area (data summarized in Oehlmann *et al.*, 1998a, and Figure 14). At VDSI values up to 4.00 in dogwhelk populations and ambient TBT concentrations < 2.0 ng Sn \( l^{-1} \), all females are still capable of reproducing, while the ISI values in sympatric periwinkle populations range from 0 to 0.40, but normally not above 0.30. At highly contaminated sites with TBT concentrations > 2.0 ng Sn \( l^{-1} \), the VDSI exceeds 4.00 and *Nucella* populations become endangered due to the progressive sterilization of females. It is in this contamination range that ISI values begin to increase. Where periwinkle populations exhibit an ISI of 0.50 or more, all females in a sympatrically living dogwhelk population are found to be sterile and the *Nucella*
population will soon be eliminated. The literature contains no reports of dogwhelk populations in areas where *Littorina* populations attain ISI values above 1.0.

**Figure 13.** *Littorina littorea*. Aqueous TBT concentration-effect relationships for the ISI:

\[ y = \frac{3.35}{1 + e^{-(x-18.3)/4.73}} \]

\( n = 34 \) samples from 27 stations, \( r = 0.975, p < 0.005 \).

![Graph of Intersex Index (ISI) vs. ng TBT-Sn/L in ambient water](image1)

**Figure 14.** Relationship between the Intersex Index (ISI) in *Littorina littorea* and the Vas Deferens Sequence Index (VDSI) in sympatrically living populations of *Nucella lapillus* (data updated from Oehlmann *et al.*, 1998a) with calculated correlation:

\[ y = 0.132 + 0.0039 e^{0.913x} \]

\( n = 92 \) samples from 91 stations, \( r = 0.814, p < 0.005 \).

![Graph of Intersex Index (ISI) vs. Vas deferens sequence index (VDSI)](image2)
The species differences in TBT sensitivity between *Littorina* and *Nucella* have to be considered when interpreting intersex assessment criteria of periwinkle populations, since the ultimate objective is not just to protect *Littorina* populations but the entire coastal ecosystem.

Table 1 provides a summary of the effects of TBT on the reproductive system and breeding capabilities of female *Littorina littorea* and sympatrically living *Nucella lapillus* at given ISI values.

**Table 1.** 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*.

<table>
<thead>
<tr>
<th>ISI value</th>
<th>Biological TBT effects</th>
<th>Aqueous TBT concentration (ng Sn l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.10</td>
<td>No adverse effects at the individual or population level</td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td>0.10 – &lt; 0.30</td>
<td>Single sterile snails may occur in populations, but no adverse effects at the population level</td>
<td>≤ 2.0</td>
</tr>
<tr>
<td>0.30 – &lt; 0.50</td>
<td>Up to 100% sterility in sympatrically living <em>Nucella</em> populations, but little or no effect on reproductive capability in <em>Littorina</em> populations</td>
<td>&gt; 2.0 – 10</td>
</tr>
<tr>
<td>0.50 – &lt; 0.70</td>
<td><em>Nucella</em> populations are unable to reproduce; some sterile female <em>Littorina</em>, but effects on periwinkle populations are generally negligible</td>
<td>&gt; 10 – 12</td>
</tr>
<tr>
<td>0.70 – &lt; 1.20</td>
<td><em>Nucella</em> populations unable to reproduce; up to 30% sterile females in <em>Littorina</em> populations</td>
<td>&gt; 12 – 15</td>
</tr>
<tr>
<td>1.20 – 2.50</td>
<td><em>Nucella</em> populations expired; incidence of sterile females in <em>Littorina</em> populations 30–80%</td>
<td>&gt; 15 – 23</td>
</tr>
<tr>
<td>&gt; 2.50</td>
<td><em>Nucella</em> populations expired; complete cessation of breeding in <em>Littorina</em> populations</td>
<td>&gt; 23</td>
</tr>
</tbody>
</table>

### 5 SUMMARY GUIDE TO INTERSEX MEASUREMENT WITH NOTES ON SAMPLING AND HANDLING

Experience has shown that consistent results can be obtained with relatively small samples of 30 to 40 adults, collected randomly during low tide, by hand, from the substrate between spring low water and mid-tide levels. In areas highly contaminated by TBT and at sites with a general, high exposure to other environmental contaminants, such as harbour areas, it can be difficult to obtain the recommended number of individuals so that sample numbers have to be reduced.

Wherever possible, the largest specimens with eroded shells, which are often covered by barnacles and/or sedentary polychaetes (e.g., *Spirorbis* sp.), should be excluded from sampling since older specimens may exhibit an intersex intensity which reflects an earlier, and possibly higher, level of contamination. The most suitable age classes for the analyses are periwinkles that have just become sexually mature (1.5 years or more). Normally, these specimens have shell heights between 15 mm and 25 mm, depending on the location.

In the laboratory, samples for biological measurements should be analysed as soon as possible, but not later than seven days after sampling, according to the following protocol (adapted from the JAMP guidelines of the OSPAR Commission). All samples containing live specimens should be transported to the laboratory in non-contaminating containers. If necessary, cooling facilities during transport and storage should be employed.
*Littorina* has to be narcotized for at least 2 hours in 7% MgCl₂ in distilled water (for Atlantic and North Sea samples; probably lower concentrations for estuarine and Baltic samples, according to the salinity at the sampling sites) to achieve a maximum relaxation, in order to simplify the measurement procedure and to provide more reproducible results. Before breaking the shell with a bench vice, individual measurements for shell heights should be made to the nearest 0.1 mm with a vernier calliper. Specimens parasitized by trematodes or other endoparasites should be excluded from further analyses because these can affect, and may disrupt, endocrine functions, thus also influencing intersex intensities.

Under the dissecting microscope, the following measurements should be undertaken for *Littorina littorea* (with an accuracy of 0.1 mm for organ extensions), in females only:

a) intersex stage (according to Figures 6 to 10, see Section 3.1, above);

b) length of the prostate gland.

On the basis of these measurements, the following calculations should be performed in order to determine intersex intensities and to estimate the possible interference with the reproductive capability of *Littorina* populations:

a) Intersex Index (ISI) as the mean value of the intersex stages in the females in a population and as the primary parameter (see Section 3.2.2, above);

b) Mean Female Prostate Length (FPrL) as the secondary parameter (see Section 3.2.3, above).

Additional measures such as the intersex incidence and proportion of sterilized females can be used, depending on the TBT exposure level in the study area (see Section 3.2, above).

Quality assurance is an important aspect of the analyses. Any deviations from the protocols should be recorded and assessed by the laboratory manager for their potential to influence the results. All determinations should be carried out by trained staff working to defined protocols. Within single laboratories, calibration exercises should be performed on a regular basis to compare the performance of different analysts. An example is given in Annex 1, comparing the data from three observers examining the same animals from a moderately polluted site on the German Baltic Sea coast. Although there is some variation in measurements, the overall results with calculations of the FPrL and scoring of intersex stages are not significantly different.

International laboratory performance studies of imposex and intersex measurements in *Nucella lapillus* and *Littorina littorea* are available through QUASIMEME (Quality Assurance of Information for Marine Environmental Monitoring in Europe) and provide a formal framework for external quality assurance (Davies et al., 1999). The QUASIMEME office is situated at the FRS Marine Laboratory, Aberdeen, Scotland (internet homepage: [www.quasimeme.marlab.ac.uk](http://www.quasimeme.marlab.ac.uk)). An example of a protocol (data sheet) as used by QUASIMEME for the assessment of intersex intensities in periwinkle populations is provided in Annex 1.
REFERENCES


ANNEX 1

INTRALABORATORY COMPARISONS OF INTERSEX MEASUREMENTS

<table>
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<th>Specimen no.</th>
<th>Shell height (mm)</th>
<th>Sex</th>
<th>Observer 1 Female prostate (mm)</th>
<th>Intersex stage</th>
<th>Observer 2 Female prostate (mm)</th>
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no. males: 10
no. females: 20
ISI: 1.55
FPrL: 1.32
Intersex incidence (%): 95.0
Sterile females (%): 35.0
No. 1  Cadmium and lead: Determination in organic matrices with electrothermal furnace atomic absorption spectrophotometry
No. 2  Trace metals in sea water: Sampling and storage methods
No. 3  Cadmium in marine sediments: Determination by graphite furnace atomic absorption spectroscopy
No. 4  Lipophilic organic material: An apparatus for extracting solids used for their concentration from sea water
No. 5  Primary production: Guidelines for measurement by 14C incorporation
No. 6  Control procedures: Good laboratory practice and quality assurance
No. 7  Suspended particulate matter: Collection methods for gravimetric and trace metal analysis
No. 8  Soft bottom macrofauna: Collection and treatment of samples
No. 9  Sediments and suspended particulate matter: Total and partial methods of digestion (videotape available)
No. 10 Organic halogens: Determination in marine media of adsorbable, volatile, or extractable compound totals
No. 11 Biological effects of contaminants: Oyster (Crassostrea gigas) embryo bioassay
No. 12 Hydrocarbons: Review of methods for analysis in sea water, biota, and sediments
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No. 37 Biological effects of contaminants: Use of intersex in the periwinkle (Littorina littorea) as a biomarker of tributyltin pollution
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