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Biological effects of contaminants: Use of imposex in the dogwhelk (*Nucella lapillus*) as a bioindicator of tributyltin pollution

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Abstract

This document describes a method for detecting contamination of the marine environment by tributyltin (TBT) using a sensitive neogastropod, the dogwhelk *Nucella lapillus* (L.), as a bioindicator. Exposure of female *N. lapillus* to TBT induces masculinization; this induction of masculinization has been termed 'imposex'. The indices that have been employed to measure imposex in *N. lapillus* are described here, together with a brief account of the biology of this organism.

Keywords: tributyltin, TBT, bioindicator, dogwhelk, *Nucella lapillus*, imposex, biological effects of contaminants

1 INTRODUCTION

Anti-fouling paints are applied to the hulls of ships and small boats to prevent the attachment of sedentary organisms, such as barnacles, tubeworms, and seaweeds. In the past, the effectiveness of these paints was usually based on the toxicity of copper, but their efficacy was much improved in the early 1960s with the introduction of formulations containing tributyltin (TBT) compounds. TBT-containing paints rapidly gained popularity, especially in the pleasure boat industry, and by the 1980s usage had become so widespread that high levels of TBT leachate began to be recorded in coastal waters, often exceeding 1000 ng l^{-1} in harbours and marinas. During the same period, various pathological conditions attributable to TBT exposure were discovered in non-target organisms, including shell malformation in farmed oysters. The realization that control of the usage of TBT was necessary to protect oyster stocks led to French legislation in 1982. This prohibited the use of TBT paints on small vessels (under 25 m in length) and, because of its effectiveness, this restriction has formed the basis of subsequent legislation worldwide.

One of the first impacts of TBT exposure to be described, but not attributed, was the masculinization of female neogastropod snails. This phenomenon was first noticed in the dogwhelk, *Nucella lapillus* (L.), around 1970 (Figure 1A) in Plymouth Sound (Blaber, 1970), and later in the American mud-snail, *Nassarius obsoletus* (Say), in Long Island Sound (Smith, 1971). Smith (1971) coined the term 'imposex' to denote a 'superimposition of male sex organs onto females'. Firm evidence of its link with TBT pollution did not appear, however, until ten years later (Féral, 1980; Smith, 1981a, 1981b), and it was not until methods for organotin analysis had been refined that the remarkable sensitivity of the imposex response became apparent.

Early field surveys revealed that imposex was identifiable in *N. lapillus* in virtually all populations around the UK, even those far distant from possible sources of TBT pollution (marinas, harbours, ferry ports and shipyards; later, salmon farm cages were added). Significantly, it was found that the degree of female masculinization increased markedly close to TBT sources. Clearly, the intensity of imposex expression could be used as a bioindicator of TBT pollution even in those areas where the concentration of TBT was below that which could be measured by chemical means (the detection limit for tin using graphite furnace atomic absorption is about 0.2 ng Sn l^{-1}).

This paper describes the indices that have been employed to measure imposex in *N. lapillus*, as a means both to assess the extent of pollution and to monitor the effectiveness of the legislation restricting TBT paint usage introduced for UK waters in 1987. Several neogastropod species have been used as TBT indicators, but around Europe *N. lapillus* has been the main species of choice, not only because it is readily available and the best known in terms of its ecology and general biology, but also because it has proved to be the most TBT-sensitive. Imposex is a genital abnormality of the female. When developed to an advanced state in *N. lapillus*, it affects the development, form, and functioning of the female reproductive system and, hence, breeding activity. The phenomenon is relatively simple to measure and, to be fully exploited as a field bioindicator, freshly-collected live samples and some knowledge of the species' biology are the two basic requirements. Relevant aspects are briefly considered here. Citations have been kept to a minimum: full accounts of the observations on which this description is based will be found in the papers by Bryan *et al.* (1986, 1987, 1988), Bryan and Gibbs (1991), Gibbs and Bryan (1986, 1994), and Gibbs *et al.* (1987, 1988, 1991) given in the reference list. Concentrations expressing TBT as Sn are used; they can be converted to TBT by multiplying by 2.44.

2 THE DOGWHELK, *NUCELLA LAPILLUS*

2.1 General Biology

An excellent account of the biology of *N. lapillus* is found in Crothers (1985). *N. lapillus* has a wide geographical distribution, being found intertidally on the rocky shores of the North Atlantic from northern Russia to Portugal, Iceland and Greenland, and from southern Newfoundland to the New York region. Like all neogastropods belonging to the Family Muricidae, it is a carnivore, feeding mainly on mussels and barnacles, although a variety of other prey are also eaten, including other gastropods such as top-shells (*Gibbula* spp.). Typically, the shell is whitish but a wide variety of colour forms occur in some populations, often with complex banding patterns (see Crothers, 1985, Plate 2). When adult, the length of the shell is generally 25–35 mm, but this varies with shape and habitat. Much of the morphological variation between populations is attributable to the species' limited powers of dispersion: development does not include a free-swimming, planktonic phase (see below) and since adults crawl only short distances, individuals spend their entire existence (unless dislodged) on the same patch of shore.

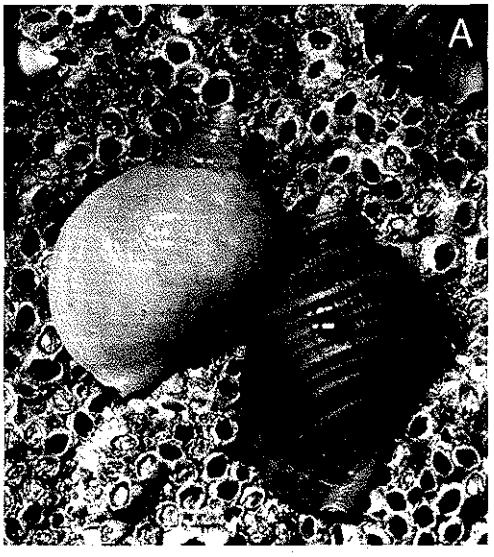
Marked individuals of *N. lapillus* have been observed to survive at least six years (Feare, 1970a), but it is likely that some live much longer, possibly a decade or more in habitats sheltered from heavy wave action. No method of accurately ageing *N. lapillus* has been discovered. The actively growing juvenile can be recognized by its thin, sharp-edged lip. One-year-olds are approaching adult length but still have a thin lip. Subsequently, the lip begins to thicken. When about three years old, the cessation of growth is often, but by no means always, marked by the laying down of a set of 'teeth' along the inside edge of the lip. In most populations, individuals appear to breed for the first time towards the end of their second year or soon after. Thus, any toothed individual can be regarded as a potential breeding adult. Males appear to mature at an earlier age than females.

In some regions, *N. lapillus* breeds throughout much of the year, but in others breeding is restricted to a few months. Spawning is accompanied by aggregative behaviour. As in all neogastropods, the sexes in *N. lapillus* are separate and fertilization is internal, sperm being transferred to the bursa copulatrix during copulation. Eggs are fertilized within the oviduct and groups of about 600 are encased by the secretions of the capsule gland (Figure 1C) to form a discrete, soft capsule which is then passed from the oviduct to the pit-like ventral pedal gland situated anteriorly on the sole of the foot. While being held in this gland, the capsule is moulded and hardened to its characteristic 'stalked vase' shape and then applied to a rock surface. The time taken to manufacture each capsule is unknown, but since partly-formed capsules are rarely discovered in the capsule gland during routine examinations, it is assumed that the process is fairly rapid, perhaps taking only an hour or two (this fact has relevance in the recognition of VDS Stage 6, see below). Spawning animals aggregate in crevices and below overhangs and, where conditions are favourable, masses comprising many hundreds of capsules are not unusual. The capsule is 7–9 mm long and 2–4 mm in diameter, but is occasionally smaller; when first laid, it is yellow and resembles a grain of wheat (Figure 1B). Embryos remain within the capsule for 3–4 months; typically, about 15–30 are destined to complete development, the rest being consumed as 'nurse' eggs. When finally emerging from the capsule through the apical hole that appears, the young, known as 'crawlaways', have the appearance of miniature adults (Figure 1B). Thus, unlike many gastropods, *N. lapillus* has no planktonic 'veliger' phase and thus lacks a dispersive larval stage in its life history.

Figure 1. *Nucella lapillus* and the effect of imposex.

Abbreviations: ac = mass of aborted capsules; c = capsule gland

A. Adults.



B. Egg capsules with emerging juveniles (two arrows).

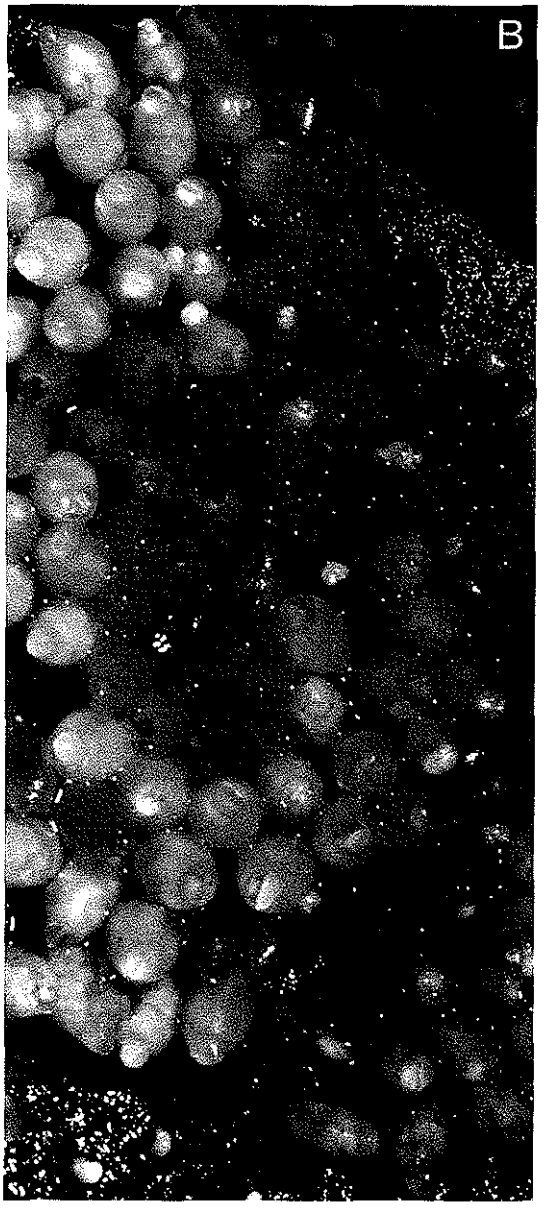


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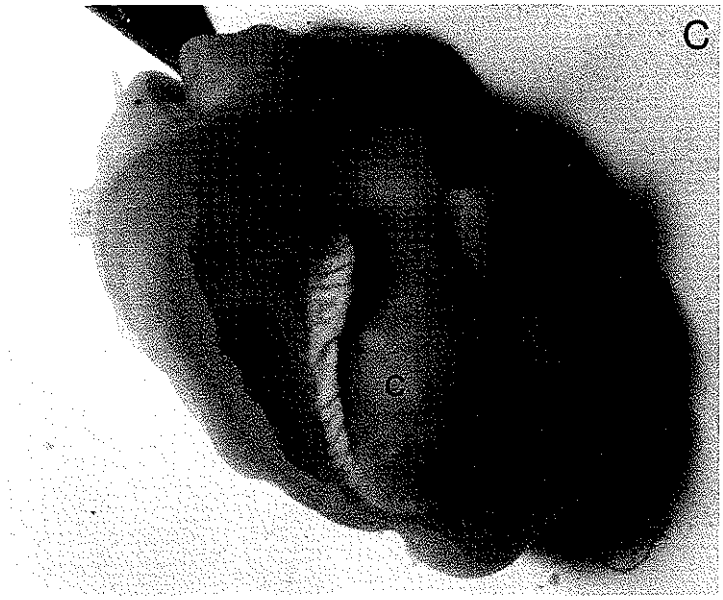
Figure 1. Continued.

Abbreviations: ac = mass of aborted capsules; c = capsule gland

C. Females removed from the shells showing the appearance of the capsule gland when normal.



D. Females removed from the shells showing the appearance of the capsule gland when containing a large, dark-coloured mass composed of aborted capsules.

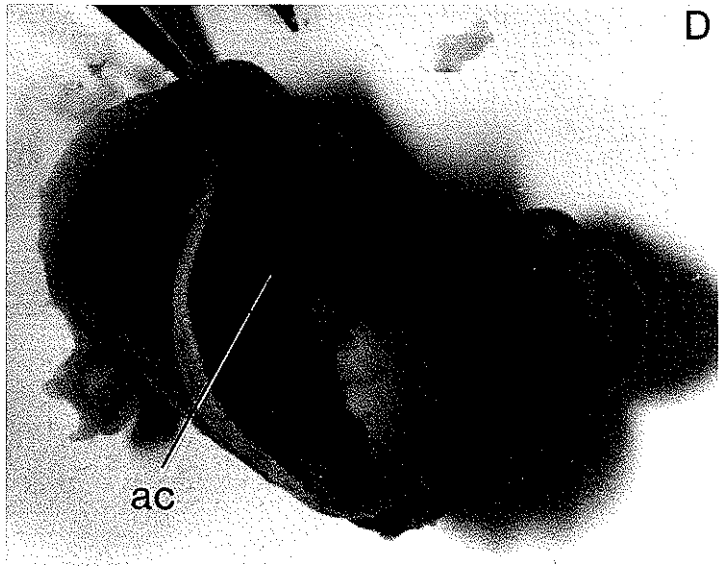


Figure 1. Continued.

E. Aborted capsules stacked in a mass (approximately 10 mm long) removed from the capsule gland of a sterilized female.

2.2 Reproductive Features

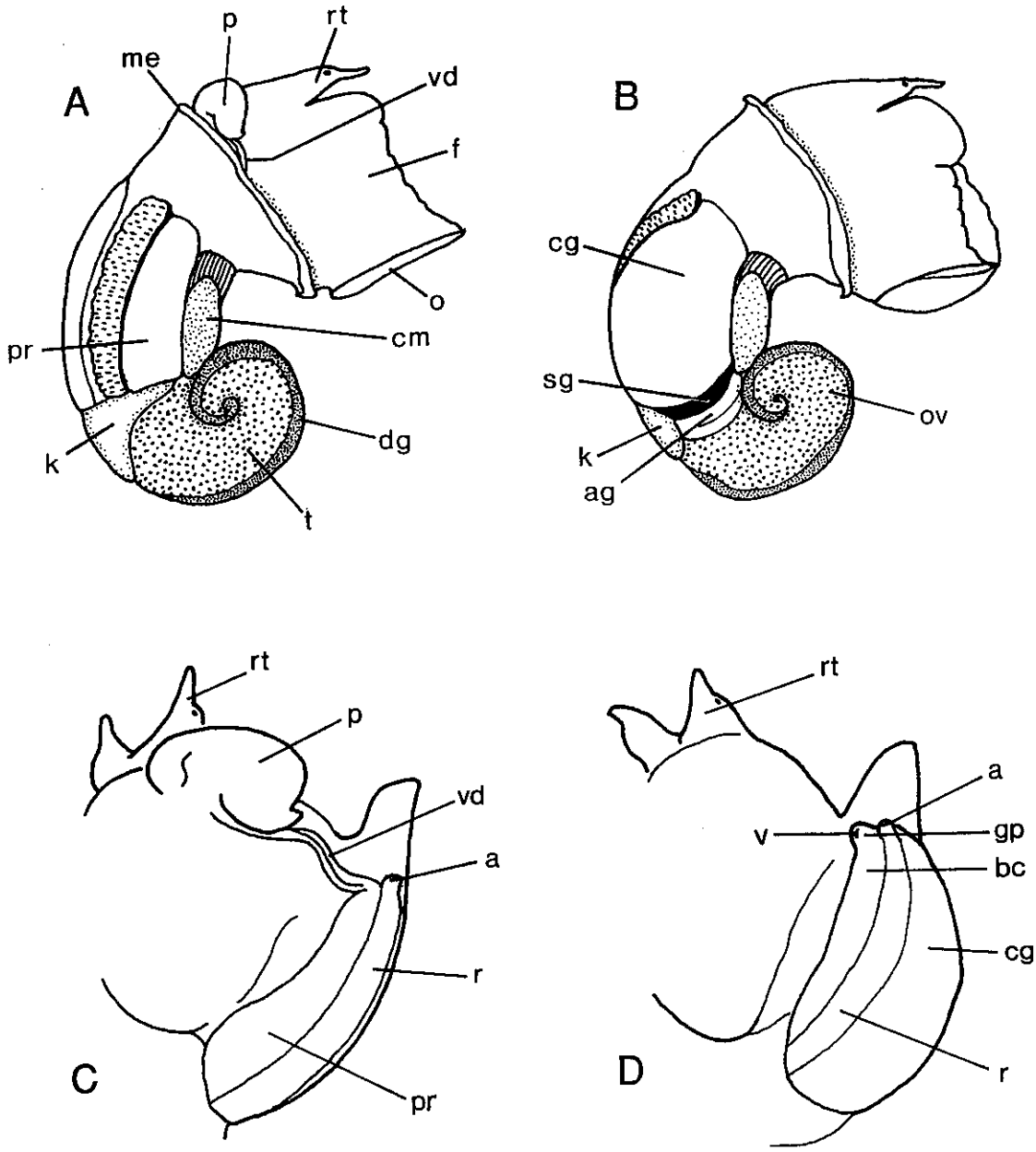
Routine sexing

The sexes cannot be distinguished from shell characters, but live sexing is possible if animals are narcotized (see below). For a complete examination of imposex, the body must be extracted from the shell. This is most easily achieved by cracking the shell in a bench vise and then detaching the large columella muscle, either by gently teasing, or cutting, at its point of attachment to the shell.

The external body features of males and females are illustrated in Figure 2A–B. Before the advent of TBT paints, the sexing of individuals was a routine simply of determining the presence or absence of a penis. At present, the masculinization of females frequently progresses to the stage where the size of the female penis approaches that of the male and thus even penis size is not a character that can be used to reliably separate the sexes in many populations; instead, secondary sex characters have to be identified. The most convenient feature by which to distinguish a female is the presence of a sperm-ingesting gland behind the capsule gland (Figure 2B); this gland appears as a transverse band of dark tissue, usually brown but it may be reddish or black. Males can be identified by the presence of a vesicula seminalis on the inner part of the visceral coil; when sexually active, this portion of the vas deferens acts as a storage organ for sperm, becoming swollen and pearly white in colour. The colours of the testis and ovary are somewhat variable depending on age and sexual condition. Both have a cream-yellow or orange colour when mature but, when developing, the testis is mustard yellow, and the ovary translucent grey. Both become brownish in old age.

Figure 2. The external features of a mature male (A, C) and mature female (B, D) after shell removal (A, B) and with mantle cavity roof cut longitudinally (C, D).

Abbreviations: a = anus; ag = albumen gland; bc = bursa copulatrix (site of); cg = capsule gland; cm = columella muscle; dg = digestive gland; f = foot; gp = genital papilla; k = kidney; me = mantle edge; o = operculum; ov = ovary; p = penis; pr = prostate; r = rectum; rt = right tentacle; sg = sperm-ingesting gland; t = testis; v = vulva; vd = vas deferens



Ideally, a sample needs to be composed of equal numbers of the two sexes, but in practice the sex ratio often deviates markedly from unity, sometimes significantly so (see Feare, 1970b). Evidence suggests that females commonly outnumber males and that the proportion of females increases with age, a feature attributed to differential mortality of the two sexes. This trend is reversed in populations suffering acute imposex (see below).

Non-sacrificial sexing

In some circumstances, it may be advantageous to sex animals without resorting to extracting them from their shells. This can be done by determining the presence or absence of a ventral pedal gland, a pouch-like cavity on the sole of the female foot which is used during egg capsule deposition. The following technique has proved highly effective. Anaesthetize specimens in $MgCl_2 \cdot 6H_2O$ (75 g in one litre of distilled water) for 2 hours to obtain full relaxation. Grip the operculum with forceps and, by gentle pulling, ease the body out of the shell sufficiently so as to be able to examine the sole of the foot. Secure the operculum against the shell using a thumbnail and place under a stereomicroscope. Examine the sole behind the head region: in females the ventral pedal gland appears as a transverse slit behind the accessory boring organ (which is common to both sexes).

Genital systems

Descriptions are given in Fretter (1941) and Oehlmann *et al.* (1988). The anterior parts of the male and female tracts are sited on the right side of the mantle (= pallial) cavity and are exposed by cutting the thin tissue forming the 'roof' of the mantle cavity along the length of the medial margin of the capsule gland. Small scissors of the angled 'iris' type are best for this job. They can also be used to gently move accumulations of mucus across the floor of the mantle cavity when the often copious secretion of this material obscures a view of the vas deferens and genital papilla, for example.

Male

A simple duct, the vas deferens, carries sperm from the testis (which, like the ovary, forms part of the visceral coil) to the penis situated on the head behind the right tentacle (Figure 2). The proximal section of this duct on the inner part of the visceral coil is convoluted forming the vesicula seminalis, a storage organ for mature sperm (Figure 3A). The middle section lies within the mantle cavity and is referred to as the prostate, recognizable by its swollen, highly glandular structure. The distal portion, termed the pallial vas deferens, traverses the floor of the mantle cavity from the prostate to the base of the penis, continuing as the penial duct to its tip. In the adult, the pallial vas deferens is a narrow sub-surface tube. It develops in the juvenile by infolding of the epithelium, first appearing as a shallow groove which subsequently deepens, the edges of the groove make contact and fuse together, and the tube so formed sinks below the surface (Figure 4). The line of fusion can be traced by a thin streak remaining on the surface.

Two features concerning the formation of the pallial vas deferens need to be noted: (1) the epithelial infolding does not occur simultaneously along the whole length: instead, infoldings appear adjacent to the developing prostate and the developing penis and migrate towards each other, eventually fusing about half way (some variation in this process has been noted: in some individuals/populations, infolding has a single origin at the site of the penis); (2) the sequence of infolding is similar when the vas deferens is superimposed onto the female in imposex and can be used to score the progress of the masculinization process (see below).

Figure 3A. Diagram of the male genital tract.

Abbreviations: p = penis; pd = penial duct; pr = prostate; t = testis; vd = vas deferens; vs = vesicula seminalis

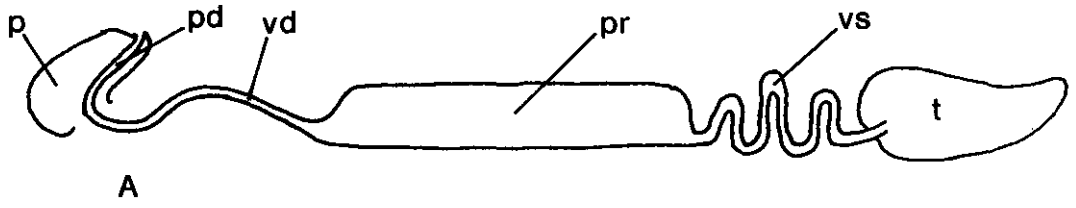
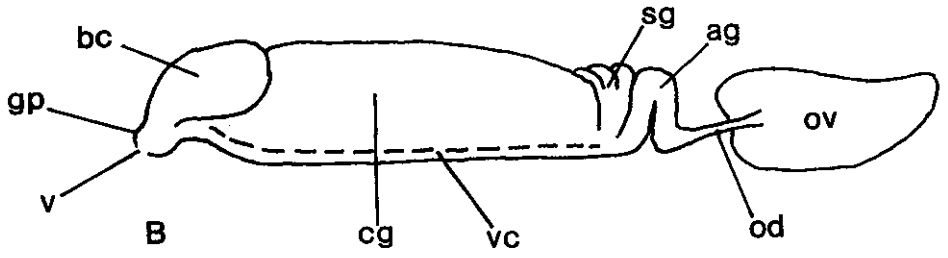


Figure 3B. Diagram of the female genital tract.

Abbreviations: ag = albumen gland; bc = bursa copulatrix; cg = capsule gland; gp = genital papilla; od = oviduct; ov = ovary; sg = sperm-ingesting gland; v = vulva; vc = ventral channel

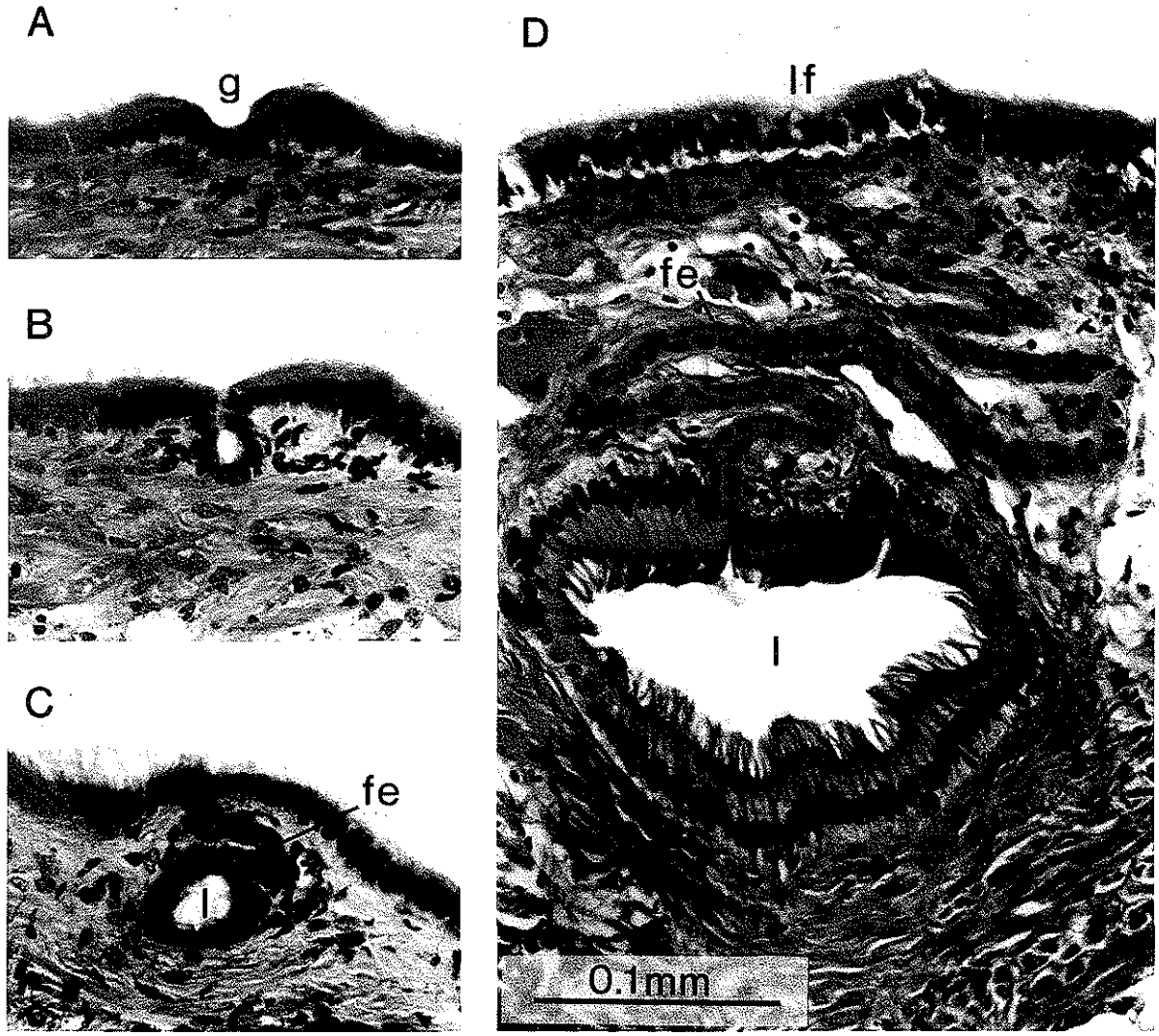


Female

The oviduct structure is more complex since various sections are modified to perform the different functions associated with capsule production. Fertilization is internal: during copulation, sperm are deposited in the bursa copulatrix and then carried backwards along the ventral channel of the capsule gland (Figure 3B). Eggs are carried forwards from the ovary via the oviduct to the albumen gland where they are mixed with secretions and fertilized before passing into the capsule gland. Here protein and mucoid material are secreted to encase the egg mass and the capsule is then expelled via the vulva to the mantle cavity, passing forwards to the sole of the foot and ventral pedal gland.

Figure 4. Stages in the development of the male vas deferens by infolding of the mantle cavity floor epithelium. A, B: initial infolding to form a shallow groove. C, D: fusion of the edges gives rise to a closed duct, which sinks below the surface.

Abbreviations: fe = fused epithelium; g = groove; l = lumen of duct; lf = line of fusion on surface of mantle cavity floor



3 THE IMPOSEX RESPONSE

3.1 Definition, Development, and Effect

The term 'imposex' is now used exclusively to describe the masculinizing effect of TBT compounds on female gastropods. This usage implies the active intervention of an external agent causing an unnatural degree of masculinization: the presence of a vestigial penis on a female should not be identified as imposex *per se* unless corroborative evidence links the condition to TBT pollution. Conclusive historical evidence that imposex is a modern phenomenon has been documented (see Gibbs and Bryan, 1994); its specificity is still being questioned (see Evans *et al.*, 1995), but no other agent with a widespread marine application has been shown to produce masculinization on the scale demonstrated for tributyltin compounds. Evidence suggests that imposex results from hormonal imbalance (see Spooner *et al.*, 1991).

The superimposition of male genitalia onto the female from its initial phase to the advanced stages involving gross modification of the female tract is, in effect, a continuum and the end result is dependent on the degree of TBT exposure. However, it is possible to identify steps in its progress that can be used to score the intensity of its expression. Six main stages, based primarily on the development of the vas deferens, are readily identifiable in *N. lapillus* using a stereomicroscope. These are outlined in Figure 5; scanning electron micrographs of specimens exhibiting these stages are found in Oehlmann *et al.* (1991).

Stage 0 Normal female, no male characters visible.

Stage 1 Appearance of an infolding of the mantle cavity epithelium adjacent to the genital papilla (start of formation of proximal section of vas deferens).

Stage 2 Appearance of a short infolding of the epithelium close to the site of penis base (start of formation of penial duct and distal section of vas deferens).

Stage 3 Proximal and distal sections of the vas deferens extend forwards and backwards, respectively; typically, a penis develops from a low ridge to a measurable protusion of recognizable shape during this stage.

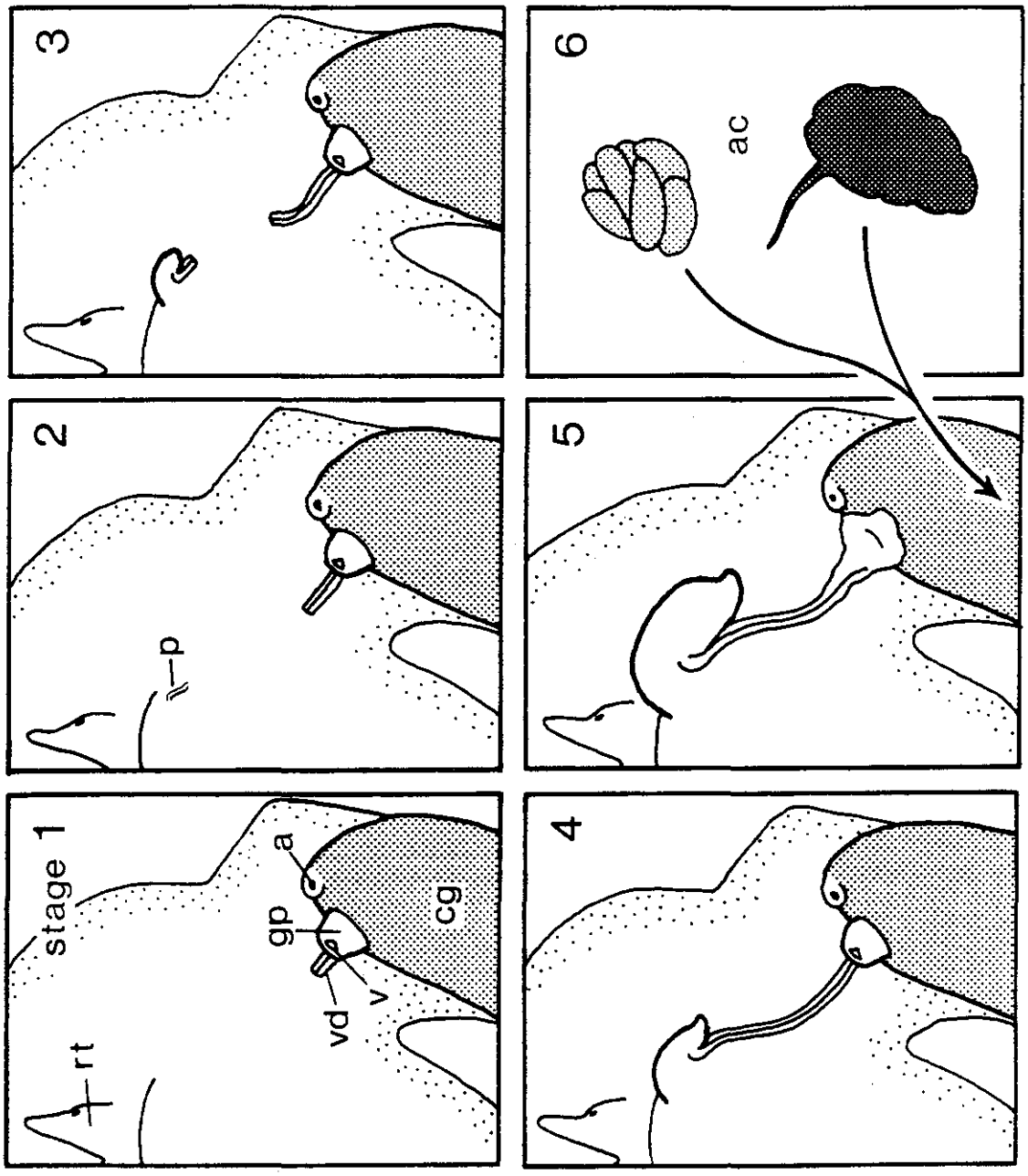
Stage 4 Proximal and distal sections of the vas deferens are now fused to form a complete duct from the penis tip to the base of the genital papilla; penis of variable size; normal breeding activity is still possible.

Stage 5 Proximal section of the vas deferens has become 'overdeveloped', with its tissue overgrowing the genital papilla to the extent that the vulva is highly constricted or not visible; nodules of hyperplasic tissue and/or translucent 'blisters' are often present; blockage of the oviduct inhibits capsule release; female is now sterile.

Stage 6 Capsules, or capsular material, can be detected within the capsule gland when the latter is opened by a longitudinal cut: a single capsule may be present or a few to many capsules may be loosely or tightly compressed together to form a soft, translucent aggregation or a hardened, dark-coloured mass (Figure 1E).

Figure 5. Six stages in the development of imposex from its initiation (Stage 1) to sterilization by oviduct blockage (Stage 5) and the later accumulation of aborted capsules (Stage 6). Numbering of the stages follows that of the Vas Deferens Sequence described in the text.

Abbreviations: a = anus; ac = accumulation of aborted capsules; cg = capsule gland; gp = genital papilla; p = penis; rt = right tentacle; v = vulva; vd = vas deferens



Notes on the recognition of the stages

Stage 1 The initial infolding occurs ventral to the genital papilla and takes the form of a shallow gutter (Figure 4). Mucus often accumulates here and this needs to be gently scraped to one side. In some populations, this stage appears to be absent.

Stage 2 When first appearing, the penial duct/distal vas deferens is very short and shallow; if not immediately visible, mucus removal and/or a change in the direction of illumination may be necessary to determine whether or not it has developed.

Stages 3/4 These stages are usually easy to identify because the vas deferens appears as a low ridge, the course of which is marked by the line of fusion. If the proximal and distal sections are not joined, it is scored as Stage 3; if continuous, it is scored as Stage 4. The genital papilla is still a distinct, rounded dome with a terminal vulva.

Stage 5 The extent of the encroachment of vas deferens tissue onto the anterior oviduct is thought to be correlated with TBT dosage; consequently, transitional stages between Stage 4 to complete occlusion of the vulva are encountered. In adults exposed to increasing TBT pollution, there is evidence that occlusion can be a gradual process taking a year or more during which period the size of the capsule that can be extruded is probably slowly diminished and the reproductive capacity likewise reduced. Such examples require a subjective judgement as to whether the overgrowth is sufficient to inhibit capsule release. Frequently, the malformation is such that there is no sign of papilla nor vulva, leaving little doubt as to the sterility of the female. This condition was invoked when females were reared to maturity (two years) at a TBT level of just 2 ng Sn l^{-1}; in these same females, the vas deferens tissue was found to have invaded the anterior oviduct, prostatic tissue replacing the bursa copulatrix and displacing the capsule gland (Gibbs *et al.*, 1988, Figure 2C). The extent of internal modification cannot be judged from the surface morphology, only by serial sectioning which, for routine sampling, is unlikely to be a practical option. However, the formation of hyperplasic nodules and translucent blisters over the bursal region (Gibbs and Bryan, 1986, Figure 3) can be taken to indicate gross modification of the oviduct and blockage.

Stage 6 The presence of aborted capsules within the oviduct is easily determined by making a longitudinal incision of the capsule gland. The process of capsule formation in the unaffected female appears to be quite rapid and is rarely observed in routine examinations (see above). Thus, even a single capsule retained in the oviduct can, with a fair degree of certainty, be regarded as abnormal; but oviduct blockage is usually signalled by the presence of a few to many capsules, usually compressed into a tight mass although sometimes the capsules accumulate as a stack with little individual flattening (Figure 1E). The mass may be translucent or coloured from light brown to black: probably the capsular material both hardens and darkens with time, maybe measured in years. Large masses can be seen externally because the capsule gland wall has become thin through stretching (Figure 1D); in extreme cases, the wall ruptures and the mass is released to become fused to the interior of the shell (Gibbs and Bryan, 1986, Plate 1F). This fusion of the mass and shell must take some time, indicating that the female can survive this severe injury, but eventually it must surely cause a premature death.

A further stage in the masculinization process can be identified in that the ovary becomes transformed to a testis, with the suppression of oogenesis and promotion of spermatogenesis to the stage where sperm are produced (see Gibbs *et al.*, 1988). Some authors have suggested that this gonadal reversal should be included in the Vas Deferens Sequence (VDS) scheme as Stage 7. However, this procedure has not been adopted widely because of the difficulty in recognizing (particularly) the initial phase of the switch from oocyte to sperm production without resorting to histological sectioning. This phase appears to occur when TBT levels in water exceed 10 ng Sn l^{-1}. Although such females would appear to have undergone a complete sex reversal and it would seem possible for sperm to travel from testis to penis, it is highly improbable that they would ever function as males: all females in the same population are likely to be in the same state and, therefore, the opportunity to copulate with a breeding female would not be realized.

3.2 Measures of Expression or Intensity

There are a number of measures that can be used to express the degree or intensity of imposex development. Each measure is useful when applied to an appropriate situation, largely depending on the degree of TBT pollution prevailing in the survey area. Where TBT contamination is light (< 0.2 ng Sn l^{-1}), the proportion of penis-bearing females is a useful measure; with higher TBT levels, female penis size, both actual and relative to that of the male, is a better indicator. The Vas Deferens Sequence can be used at all levels of pollution: above 2 ng Sn l^{-1} the percentage of sterile females gives a clear indication of any reduction in breeding capability.

3.2.1 Proportion of penis-bearing females

In areas where only a fraction of the females exhibit masculinization, the most commonly used measure is the percentage of penis-bearing females. Often this is referred to as the incidence or occurrence of imposex, but clearly this is not strictly correct since some, or all, of the non-penis-bearers will certainly exhibit the earlier stages (Stages 1 and 2, above). As a rule, percentages become redundant as a measure when the level of TBT contamination reaches the level of detection by chemical means (approximately 0.2 ng Sn l^{-1}): typically all females possess a penis at this level of exposure.

In recent years, TBT levels in UK coastal waters have declined and a means of detecting the rate of change over time periods of months rather than years can be advantageous. One method used successfully in 'clean' areas is to measure changes in the extent of the penis-bearing habit in sub-adult females aged 12–18 months old (nearly adult size, but retaining a sharp-edged lip). In populations breeding for much of the year, this age group has a continual through-put of individuals and the degree of imposex in these young females will reflect TBT levels over the previous year or so. The 'reaction' time of this measure is thus shorter than that for the indices based on adult imposex, which have a time lag of several years (see below). Data relating to penis-bearing in sub-adults are illustrated in Gibbs and Bryan (1994, Figure 7).

3.2.2 Female penis size

In neogastropods, the size and shape of the penis vary according to species. In adult male *N. lapillus*, it is roughly barrel-shaped, somewhat flattened, and often presents a curved tip. The female penis has the same form. Length is the most easily determined parameter and this can be measured, with the specimen under water, from the tip of the penis to its junction with the body wall behind the right tentacle. Measurements to the nearest 0.1 mm can be made either using a piece of 1 mm-graduated graph paper placed under the leading edge (anterior) of the penis or by means of an eyepiece graticule. This parameter is a sensitive measure for imposex at its

intermediate levels of expression (Stages 3 and 4), when the major part of development of the penis proper takes place; thereafter, attention focuses on disruption of the anterior oviduct by vas deferens tissue.

Female penis size has proved to be a convenient indicator of TBT exposure. Under conditions of increasing pollution, female penis growth is promoted in both juveniles and adults, as clearly demonstrated by the results of various experimental studies, including field transplantations (Gibbs and Bryan, 1994, Figure 6), laboratory exposures (e.g., tests of specificity: Bryan *et al.*, 1988), and combinations of laboratory/field studies (e.g., 'pulse' experiments: Bryan *et al.*, 1987). However, penis size is related to body size, which is subject to wide variation depending on habitat and perhaps food type and supply. Obviously, if the degree of masculinization of different populations is being compared, the size of the female penis for one population must be expressed as a proportion of the male penis size for the same population. In comparing the relative size of the female penis with that of the male, the mean length of the former can be expressed as a percentage of the latter. In calculating the female mean, all individuals are counted, including the zero values of both non-penis-bearers (Stages 0–2) and genuinely aphallic individuals (Stages 3+, see below). Some authors have preferred to exclude such females from the mean calculation; if this principle is applied, it needs to be stated. Comparisons based simply on length do not convey any contrast in mass between, e.g., a penis of 2 mm and one of 4 mm. However, the weight (or volume) of the penis is related to the cube of its length and, therefore, the ratio

$$\frac{(\text{mean female penis length})^3}{(\text{mean male penis length})^3} \times 100$$

is used as a measure of penis expression in a population. A level of 50 % indicates that the average female penis has half the bulk of that of the male. This measure was established by Bryan *et al.* (1986) to describe the 'degree of imposex', but with the introduction of other measures it is now designated the Relative Penis Size Index, or RPSI (Gibbs *et al.*, 1987). This index is useful when imposex levels over a wide geographical region need to be compared, since variations between populations in body and penis sizes are taken into account. Preferably, samples forming part of a survey should be taken during the same season, since male penis size may vary with the spawning cycle: a seasonal enlargement of the male penis will reduce the RPSI. In practice, this seasonal variation is a only a minor source of error because many populations breed throughout the year.

The biometric relationship between penis length and penis weight was established using non-narcotized specimens. Some authors have narcotized specimens before measuring the penis (e.g., Oehlmann *et al.*, 1991; Stroben *et al.*, 1992): RPS indices obtained with narcotized *N. lapillus* are lower than indices calculated for non-narcotized specimens, and the data are not directly comparable (Huet *et al.*, 1995). If narcotization is preferred, then the relative penis sizes of the two sexes can be expressed as the Relative Penis Length Index (RPLI) calculated from uncubed mean lengths. Similarly, this index can be applied to measurements of specimens thawed after storage in a freezer, if examination in a fresh state is impracticable. However, it is important that the protocol followed be clearly explained and all samples given the same treatment.

3.2.3 Vas Deferens Sequence

Although penis development is important in the context of providing a conspicuous marker, it is unimportant in terms of the biological consequence of imposex, i.e., sterilization through oviduct blockage. This results from vas deferens formation, and this process should be the

major focus. The Vas Deferens Sequence Index (VDSI) is the mean of the stages described above. A VDSI of 4 and below indicates that all females are capable of breeding; a VDSI above 4 points to the presence of sterilized females and a population with a reduced breeding capacity; an index above 5 indicates that most, or all, females are sterilized and the population is vulnerable to extinction.

3.2.4 Proportion of sterile females

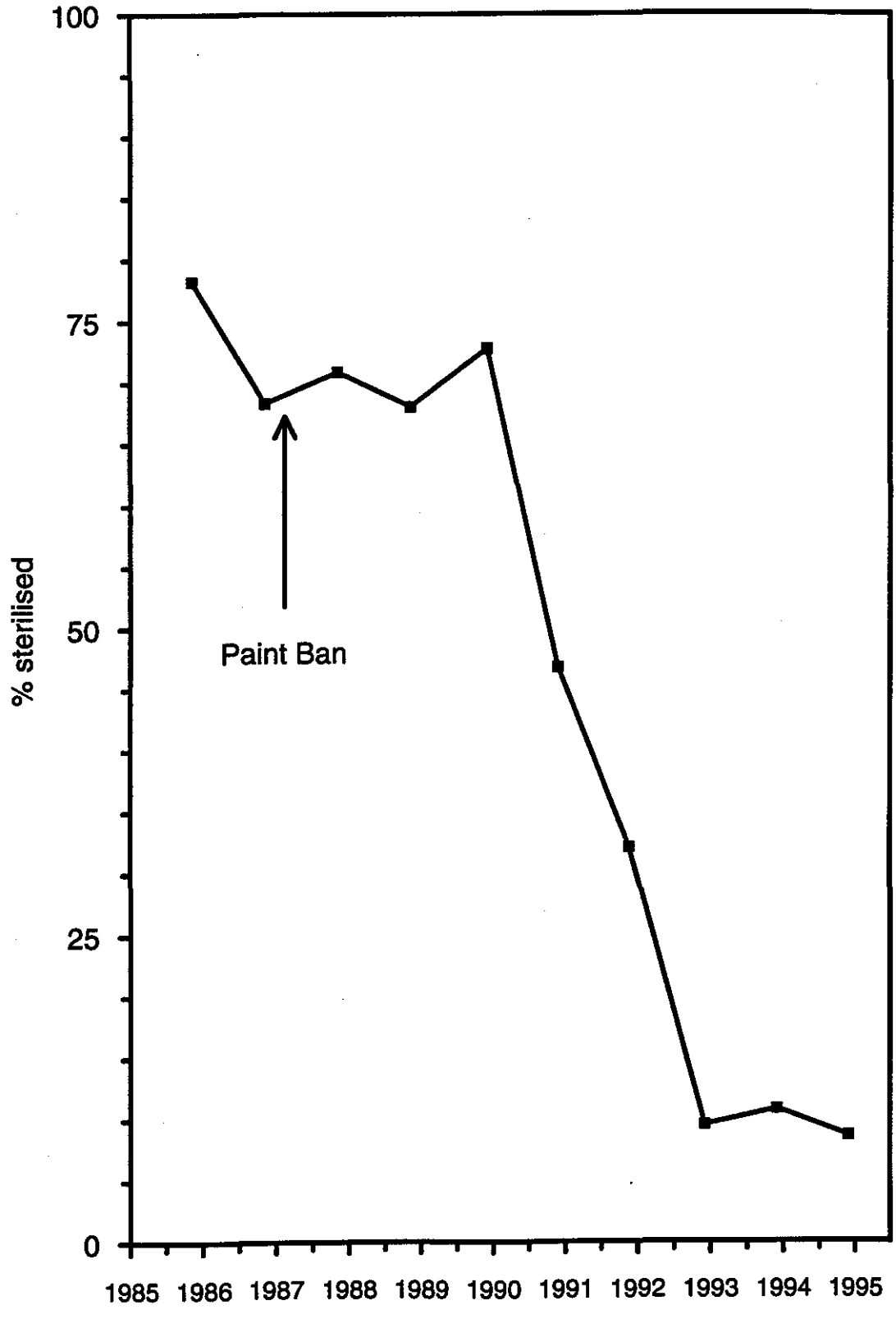
In regions where TBT pollution is high, an alternative measure that can be considered for use as an indicator of its impact is the proportion of sterile females within a population. With rising TBT levels, the intensity of imposex in adult females can increase so that the percentage of sterile individuals increases. In some circumstances, it is possible that vulnerable populations are sustained only by the progeny produced by recently matured females, which after one or two seasons are then rendered sterile. Experimentally, it has been shown that young females, reared from the hatchling stage over two years at a TBT level of about 2 ng Sn l^{-1}, can be sterilized before they reach the age of maturity at 2–3 years old and thus never breed. In the field, this state would signal the terminal decline of a population. However, providing that some females remain capable of breeding, then recovery is possible. For example, a level of 70 %–80 % sterilization was reached in a threatened population (Renney Rocks) close to the port of Plymouth (see Figure 6) but, following the amelioration of TBT pollution levels, a near-complete recovery of breeding capacity was achieved in 1995.

When the proportion of sterilized females reaches 100 %, extinction of the population is, of course, inevitable, but the time taken to disappear entirely may be many years, given the longevity of the species. The terminal phase is signalled by a preponderance of males in the population; apparently the stress imposed by the abnormalities of advanced imposex increases the mortality rate for females over that for males.

3.2.5 Variations

The principle of the VDS scheme has been applied, with variations to suit individual morphology, to various species worldwide. The VDS scheme described for *N. lapillus* above is necessarily simplified and local variations do occur. Such variations can be incorporated into an analysis, if required (see Oehlmann *et al.*, 1991; Stroben *et al.*, 1992). One variation occasionally encountered in *N. lapillus* is the absence of penis development in Stage 3+ females; the reason for this aberration is unclear, but it does not preclude VDS scoring based on the extent of vas deferens development. However, if a male lacking a penis is discovered, then the population under study is probably affected by 'Dumpton Syndrome' (DS). This syndrome is an unusual defect involving the non-development or partial development of the male genital system: an affected individual lacks a penis, or has an undersized penis, and its gonoduct (vas deferens and prostate) is incompletely developed. Importantly, this deficiency is manifest also in females exposed to TBT in which the form taken by imposex is less intense. The evidence points to DS being a genetic disorder which lessens the sterilizing effect of imposex and thereby has permitted the survival of the species in areas of high TBT pollution. DS was first noticed in a population on the southeastern coast of England, close to the ferry port of Ramsgate in Kent (Gibbs, 1993a). However, similarly deficient males have now been discovered in the Bay of Brest in northwestern France (M. Huet, pers. comm.) and it is possible that DS is a widespread, but localized, condition.

Figure 6. Percentages of sterilized females in a population close to the port of Plymouth, south Devon (Renney Rocks), illustrating recovery from a state of near-extinction following the restrictions on TBT paint usage introduced in 1987.



4 INTERPRETATION OF DATA

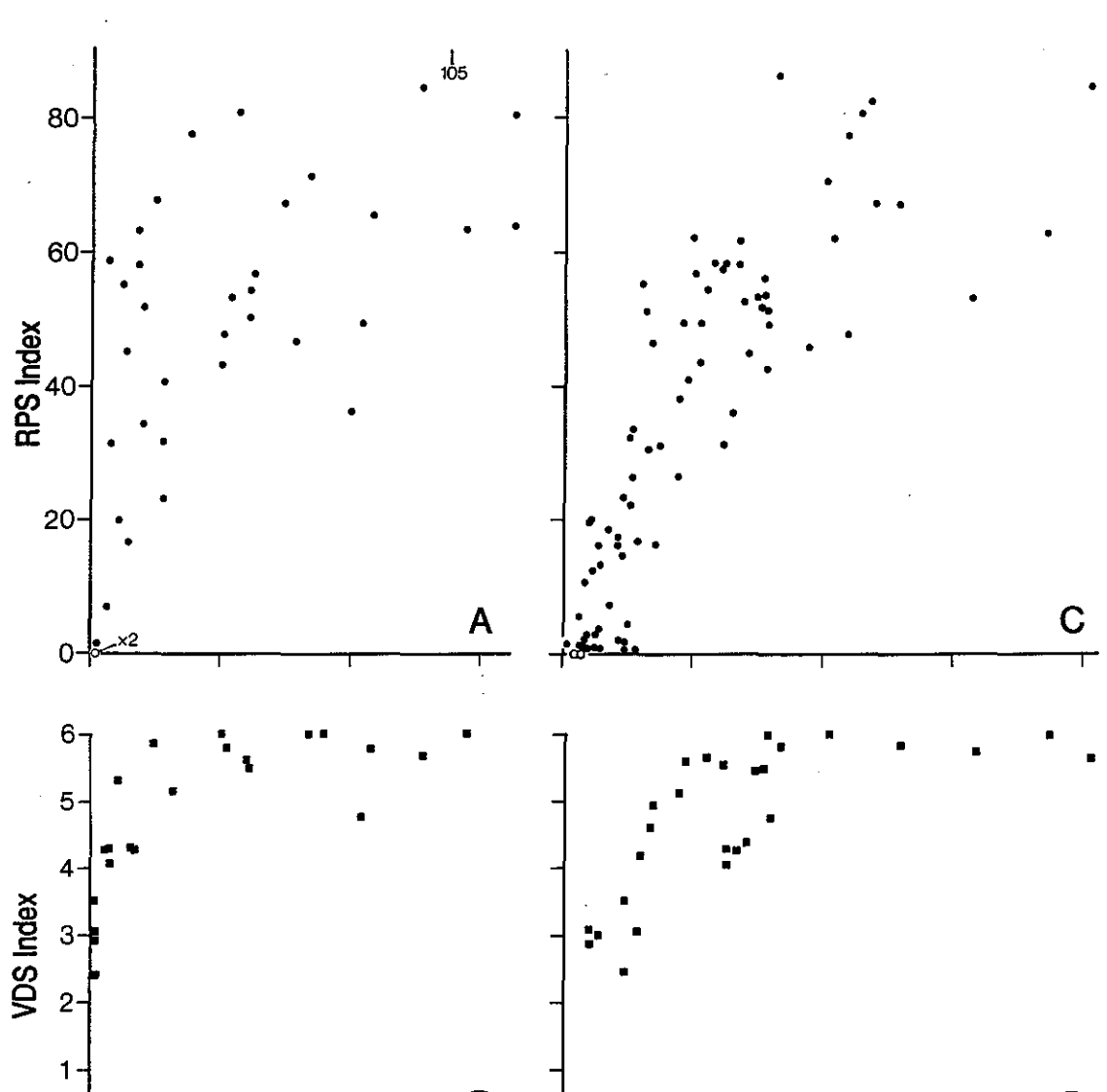
The relationships between the RPSI/VDSI values and TBT concentrations in ambient water and female body tissues are illustrated in Figure 7. As can be seen, both indices reach maximal values at water TBT concentrations of a few ng Sn l^{-1} and are correlated with tissue burdens.

Table 1 gives a summary of the effects of TBT exposure on the reproductive system of female *N. lapillus*. These observations are based mainly on data obtained from surveys of the coasts of southern England and also from field and laboratory experiments (including combinations): evidence suggests that the response is uniform throughout much, if not all, of the species' geographical range (see Gibbs *et al.*, 1991).

Table 1. Summary of the effects of TBT exposure on the reproductive system of female *Nucella lapillus* (after Gibbs *et al.*, 1988).

TBT concentration in water (ng Sn l^{-1})	Morphological modifications of genital tract	RPSI	VDSI
< 0.5	Breeding normal. Development of penis and vas deferens.	< 5	< 4
1–2	Breeding capacity retained by some females; others sterilized by blockage of oviduct as indicated by presence of aborted capsule masses.	40+	> 4
3–5	Virtually all females sterilized. Oogenesis apparently normal.	40+	5–6
10+	Oogenesis suppressed. Spermatogenesis initiated.	-	-
20	Testis developed to variable extent. Vesicula seminalis with ripe sperm in the most affected animals.	-	-
100	Sperm-ingesting gland undeveloped in some individuals.	-	-

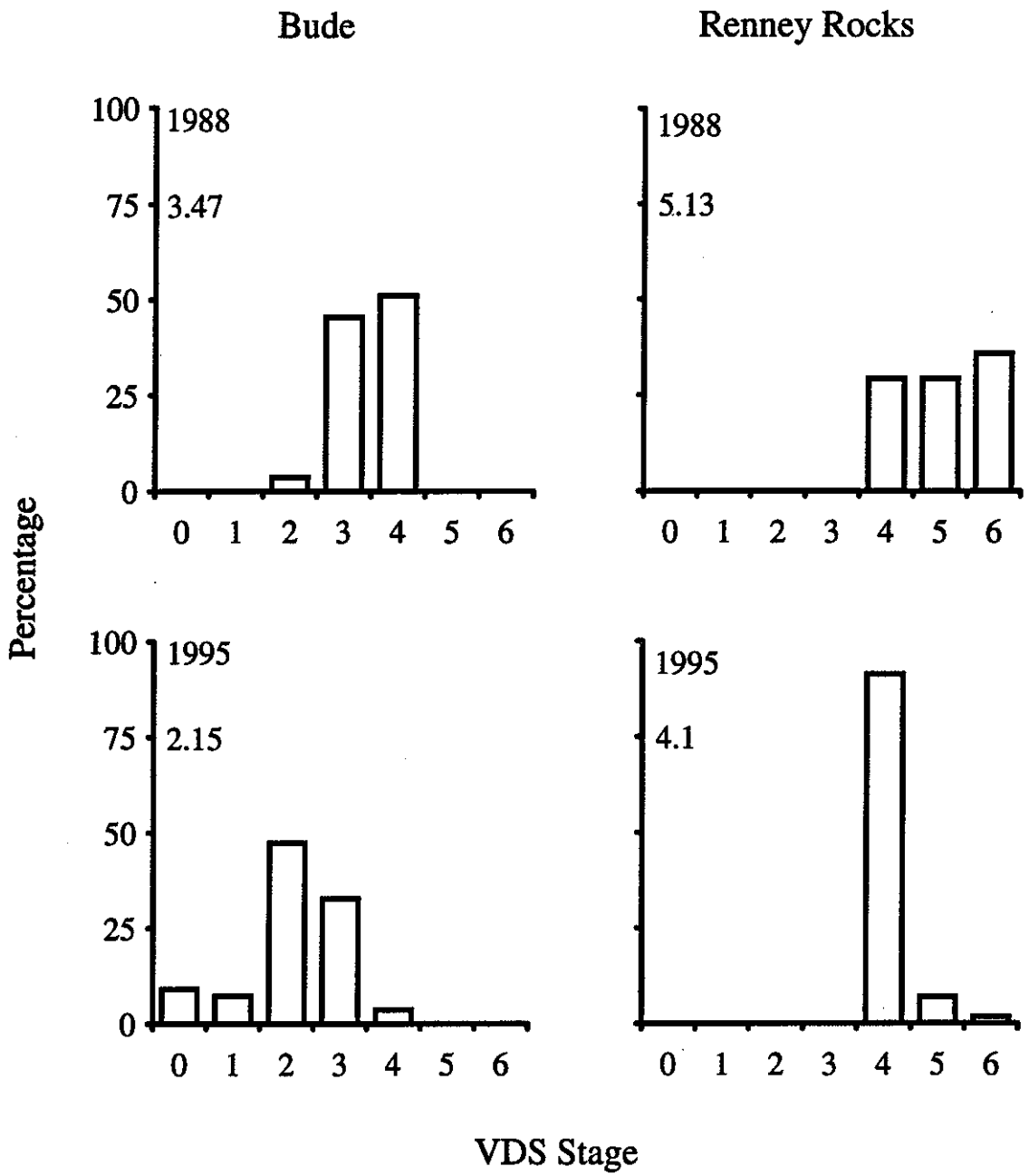
Figure 7. The relationships between the RPS and VDS indices and TBT concentrations in water and female body tissues. These samples were taken from the southwestern coast of England in 1984–1986; water and animals were collected at the same time. Open symbols are for two clean-water sites (Isle of Mull, western Scotland) where no imposex was observed.



In interpreting these morphological indicators in relation to ambient TBT concentrations, several factors relating to both the nature of imposex and the population biology of *N. lapillus* need to be taken into account. For *N. lapillus* it is known that imposex is irreversible, the intensity of its development is dose-dependent, and the most sensitive phase in the life history appears to be the late juvenile/sub-adult stage when the reproductive tract is forming. To a large extent, the intensity of imposex in the adult appears to reflect the dosage received during adolescence. Increasing TBT pollution promotes imposex in both juveniles and adults, and this increasing response is indicated by increases in the indices described above. However, during periods of declining pollution, because of its irreversibilty, imposex becomes a less sensitive indicator since the corresponding decline in its expression only becomes apparent in the indices when the more-affected females in a population are replaced by less-affected females. The rate at which this turnover of individuals occurs depends largely on the life history characteristics, especially age at maturity and longevity. The latter may vary considerably according to habitat, for example, higher mortality at exposed sites through wave dislodgement, and in sheltered inlets through crab predation (see Gibbs, 1993b).

N. lapillus females mature when two to three years old. Under conditions of declining TBT concentrations, cohorts of maturing females (one to two years old) will be progressively less affected by imposex and will be part of the adult population about a year later. However, their lessening degree of imposex will not result in a significant decline in the overall imposex level in the population indices until there has been an appreciable mortality of the more-affected, older cohorts. If a longevity of 7–10 years is assumed, it may take four or five years for any amelioration in TBT pollution to be reflected in lowered imposex levels. This temporal 'stagger' of the relationship between ambient TBT levels and the population response is well illustrated by the sterility data of the Renney Rocks population (Figure 8). The site is at the entrance to Plymouth Sound (naval dockyard, fishing harbour, marinas, anchorage for large vessels). In the years 1986 to 1990, about 70 %–80 % of *N. lapillus* females were sterilized but, after the 1987 legislation, TBT concentrations in water slowly declined so that present levels (from 1995) rarely exceed the threshold at which sterilization occurs (i.e., 2 ng Sn l^{-1}). However, the effectiveness of the paint ban was not indicated *biologically* until 1991 when the sterility rate began to drop, reaching a baseline of around 10 % in 1993 where it has remained. This dramatic recovery of a vulnerable population has been brought about by the replacement of old, sterilized females by younger animals which are no longer being sterilized before reaching maturity or soon thereafter.

Figure 8. The effect of the UK 1987 TBT-paint restrictions on the VDS profiles of two populations, one close to (Renney Rocks) and one distant from (Bude) TBT sources. Numbers shown are VDSIs: both decreased between 1988 and 1995, indicating an overall decline in the level of TBT pollution. All four profiles are based on samples of 50–60 adults collected during March to May.



Before legislation, and its consequent lessening of TBT pollution, it was usual to find that all females within any one population exhibited a similar intensity of imposex. Penis lengths were fairly uniform and VDS stages were clustered, ranging from 0–2, or 2–4, or 4–6 (at heavily polluted sites, only Stages 5 and 6 would be found). Given that imposex is irreversible, this grouping of the stages can be interpreted as a feature of either steady-state or increasing pollution. With declining pollution levels, the recruitment of newly-matured females with ever-lowering VDS scores will cause the VDS profile to shift to the left and, when TBT levels are sufficiently low, to include a wider range of stages than formerly. To illustrate this point, Figure 8 presents the 1988 and 1995 VDS profiles for the populations at Renney Rocks and Bude. At the former site, TBT pollution amelioration has resulted in fewer sterilized females and VDS Stage 4 now predominates. At Bude, where the TBT has remained at or below the level of chemical detection (~ 0.2 ng Sn l^{-1}), females that were scored VDS Stages 3 and 4 are being replaced by less-affected females, including some that show no sign of imposex (Stage 0).

5 NOTES ON SAMPLING, HANDLING, AND CHEMICAL ANALYSES

Experience has shown that consistent results can be obtained with relatively small samples of 30–40 adults (normally toothed individuals) collected at random. This number is sufficient to allow for some deviation from unity in the sex ratio. At those sites where the species is scarce or rare, sample numbers have to be fewer so as to avoid decimation of the population. Conservation of existing stocks needs to be borne in mind at all times. Note should be taken as to whether or not there is any sign of recent breeding activity, as evidenced by the presence of egg capsules or juveniles. Animals survive in good condition for a day or two if placed in a plastic bag along with damp seaweed, preferably *Fucus* spp., and kept in a cool-box.

In the laboratory, individual shell character and appearance (length, whether toothed, young, ancient, etc.) are recorded before cracking the shell in a bench vise. On extraction of the body, sex can be determined as described above. Especially in samples collected near sewage outfalls, some animals parasitized by trematodes will be encountered: these should be discarded since the parasite invades the tissues of the genital tract, including the gonad, and thereby disrupts reproductive functions. After examination, specimens can be deep-frozen if required for tissue analyses of TBT and its derivatives.

Quality assurance is an important aspect of data gathering. An example of the calibration exercises required is given in Annex 1. This compares the data of two observers examining the same animals in a sample from a 'clean' site in north Cornwall. Inevitably, there is some variation in the measurement of the penis because this organ is muscular and responds to touch. Overall, good agreement in mean penis length calculations and scoring of VDS stages can be obtained between observers given minimal tuition and some experience.

The advantages and disadvantages of imposex measurement over chemical analysis are summarized in Annex 2, which similarly outlines the case for and against using *N. lapillus* over other neogastropods.

A summary of the methods employed for the determination of TBT in neogastropod tissues and sea water can be found in Gibbs and Bryan (1994).

6 SUMMARY GUIDE TO IMPOSEX MEASUREMENT

Sampling

At sites where *N. lapillus* is common, collect 30–40 adults at random (adults can be recognized by a thickened lip which is usually 'toothed'). If the species is less than common, reduce the sample number according to availability. Check for signs of recent breeding activity, i.e., the presence of egg capsules or juveniles (thin lip, no teeth). Transport the specimens in a plastic bag containing damp seaweed (*Fucus* is good): if kept cool, they will remain in good condition for two or three days, longer if given a daily bath for about 30 minutes. It is essential that the samples are in a healthy state when examined: moribund specimens are useless.

Preliminary observations

Record the shell length. Decide which indices of penis size are needed. A Relative Penis *Length* Index (RPLI) can be based on measurements of either narcotized or non-narcotized specimens. (Narcotization involves immersion in a solution of $MgCl_2 \cdot 6H_2O$ (75 g in 1 litre distilled water); full relaxation is achieved in about 2 hours.) If a Relative Penis *Size* Index (RPSI) is required, specimens should not be narcotized.

Dissection

Break the shell in a bench vise; remove the body by detaching the columella muscle from the shell. Identify the sex of the specimen: females can be readily identified by a sperm-ingesting gland, a transverse band of dark tissue behind the capsule gland; males by a vesicula seminalis, a pearly-white, convoluted duct on the inner part of the visceral coil. Expose the mantle (= pallial) cavity floor by cutting the thin tissue forming the roof of the cavity along a line to the left of the capsule gland and parting the two flaps.

Imposex measurement/scoring

Male

Measure the length of the penis to the nearest 0.1 mm using a piece of 1 mm graph paper or a graduated eyepiece micrometer. (If no penis is present (rare), suspect Dumpton Syndrome and take note of special features (see Gibbs, 1993a).)

Female

Determine whether a penis is present: if so, measure the length using the same procedure as for the male. Score the extent of sperm duct development according to Vas Deferens Sequence (VDS). If not conspicuous, check for signs of initial stages of formation, indicated by infolding of the mantle epithelium just ventral to the genital papilla (Stage 1) and also at the site of the penis (Stage 2). The sperm duct usually develops from both sites (Stage 3), eventually joining about half-way (Stage 4). If development is complete, check whether a vulva is present allowing expulsion of capsules (still at Stage 4, female still capable of breeding), or whether it is blocked by an overgrowth of vas deferens tissue (Stage 5, female now sterilized). In either case, open the capsule gland with a longitudinal incision to determine whether aborted capsules are present (Stage 6).

Calculation of indices

Relative Penis Length Index (RPLI)

$$\text{RPLI} = \frac{(\text{mean female penis length})}{(\text{mean male penis length})} \times 100$$

An index of 50 % indicates that the mean length of the female penis is one-half that of the male.

Relative Penis Size Index (RPSI)

$$\text{RPSI} = \frac{(\text{mean female penis length})^3}{(\text{mean male penis length})^3} \times 100$$

An index of 50 % indicates that the mean bulk (mass/weight) of the female penis is one-half that of the male.

Vas Deferens Sequence Index (VDSI)

VDSI = mean of Vas Deferens Sequence stages scored

An index above 4 indicates that some females in the population are sterilized; above 5, most or all are in this condition.

Note that VDSI is the best indicator of imposex intensity, but RPL/RPS indices provide useful supplementary data.

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ANNEX 1

INTERCOMPARISON OF IMPOSEX MEASUREMENT TECHNIQUES

Nucella Lapillus. Bude population (sample collected 18 February 1995).								
			Observer 1			Observer 2		
Specimen No.	Shell length	Sex	Male penis	Female penis	VDS stage	Male penis	Female penis	VDS stage
1	27.3	m	3.6			3.6		
2	26.9	m	4.4			4.3		
3	29	m	4.1			4.1		
4	26.3	f		0	2		0	2
5	26.8	f		1.3	3		1.5	3
6	29.7	m	4			4.3		
7	23.8	m	4.1			3.9		
8	29.3	f		0	2		0	2
9	24.9	f		0	2		0	2
10	27	f		0	2		0	2
11	25	m	4.3			4.2		
12	27.7	m	4.3			4.2		
13	26.2	m	3.8			3.7		
14	30.1	f		0.8	3		0.8	3
15	25.6	f		0	0		0	0
16	24.6	m	3.9			3.8		
17	27.4	m	4.1			3.9		
18	25.2	f		0	2		0	2
19	27.6	m	4.1			4		
20	27.4	m	3.8			3.8		
21	29.1	f		0	2		0	2
22	28.7	f		0	0		0	0
23	27.8	f		0	0		0	0
24	26.5	f		1.5	3		1.5	3
25	30.6	f		1.3	3		1.4	3
26	25.4	f		0.8	3		0.9	3
27	26.3	f		0	2		0	2
28	27.5	m	4.2			4.1		
29	22.8	m	3.3			3.3		
30	27.1	f		1.2	3		1	3
31	23.2	f		0.8	3		0.9	3
32	25.2	f		0	2		0	2
33	24.5	m	4			3.9		
34	27.8	m	3.8			3.7		
35	27.2	f		1.1	3		1.1	3
Number			16	19		16	19	
Mean			3.988	0.463		3.925	0.479	
s.d.			0.283	0.583		0.274	0.604	
RPS1			0.16			0.18		
VDSI					2.11			2.11
Percentage Sterile					0			0

ANNEX 2

RATIONALE FOR USING IMPOSEX AS A BIOINDICATOR OF TBT AND *N. LAPILLUS* AS THE SPECIES OF CHOICE

Use of imposex as a bioindicator of TBT

Advantages

1) Highly sensitive: response initiated by TBT concentrations below that which can be detected by chemical means.

2) Specific: no other agent has been demonstrated to produce the full response.

3) Intensity of expression is dose related: the exposure regime can be estimated.

4) Response is visible: observers can be easily trained to score stages.

5) Process of masculinization commences early in life when penis formation (at least) provides a conspicuous marker of exposure (but see under *N. lapillus* below).

6) No sophisticated equipment is required: therefore, surveys are relatively inexpensive.

Disadvantages

1) Imposex is irreversible: intensity of expression may reflect higher TBT levels than those current at the time of sampling.

2) Can only be assessed accurately on fresh specimens.

3) The full extent of masculinization cannot be assessed until the reproductive system is fully developed; this stage is not reached in many species until the female is approaching maturity at (probably) 18–24 months old.

N. lapillus as the species of choice

Advantages

1) Response to TBT exposure is known to be uniform throughout much of the species' geographical range.

2) The species is easy to recognize: it is unlikely to be confused with any other intertidal species.

3) Intertidal: this facilitates both collecting specimens and the assessment of species abundance.

4) Wide geographical distribution: see above.

5) Entire life cycle spent in one place: development completed within capsule without a free-swimming phase; adults move only short distances from point of hatching and, therefore, their condition will be a reliable indicator of conditions prevailing at the sampling site.

6) Reproduction is affected, giving additional parameters for assessment, namely,

   a) evidence of breeding activity is obvious on the shore: development occurs over an extended period (3–4 months) within a conspicuous, readily identifiable capsule; the latter is durable and often remains attached for several weeks after hatching is complete;

   b) recognition of female sterilization is possible: consequent lowering of the reproductive capacity of the population can be gauged.

7) Animals are fairly hardy and can be transplanted between sites: they also survive well under laboratory conditions.

8) Sex ratio is about unity (except when a population is in terminal stages of decline); sex can be determined by non-sacrificial means.

Disadvantages

1) Long-lived: imposex being irreversible, the intensity of the observed extent of masculinization in older cohorts can reflect TBT levels of, maybe, 5–10 years earlier.

2) Species distribution restricted to rocky shores.

3) Does not tolerate brackish conditions.

4) Can be difficult to gauge breeding success from the abundance of juveniles since, at least on British shores, adults appear to dominate many populations (Crothers, 1985).

5) No method of ageing specimens has been devised, but the size and character of shell permit broad categorization into 'juvenile', 'sub-adult' and 'adult'.

6) High TBT sensitivity causes extinction in areas subject to high pollution levels and therefore the organisms are not available for monitoring.

Reference

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No. 24	Biological effects of contaminants: Use of imposex in the dogwhelk, *Nucella lapillus*, as a bioindicator of tributyltin pollution

Reporting protocol

- Biological TBT effects monitoring using imposex in *Nucella lapillus* -

General information

Sampling site:

Name and/or code: _____

Coordinates (e.g. GPS): _____

Anticipated exposure ☐ reference site ☐ impacted site

Approx. distance from TBT source (harbour, dock, sewage outfall, etc.) _____ km

Date of sampling: _____

Date of analysis: _____

No. of snails sampled: _____

Signs of recent breeding activity: presence of: ☐ egg capsules ☐ juveniles

Methodology used:

Analysis according to ☐ ICES TIMES series ☐ others

If others summarise deviations from TIMES methodology: _____

_____

_____

_____

Name(s) of examiner(s): _____

_____

Results

Total no. of snails analysed: _____

no. of males: _____

no. of females: _____

Mean shell height [mm]: _____

Mean male penis length [mm]: _____

Mean female penis length [mm]: _____

Vas deferens sequence index (VDSI): _____

Relative penis length index (RPLI): _____

Relative penis size index (RPSI): _____

Percentage sterile females: _____