

**DEVELOPMENT OF A PROTOCOL FOR IMPOSEX MEASURES  
ON TROPICAL NEOGASTROPOD MOLLUSCS**

by

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### ABSTRACT

Tributyltin (TBT) used in antifouling paints is toxic to many non-target organisms. A known sublethal effect of TBT to marine organisms is neogastropod imposex, i.e., the imposition of male sexual characters on functionally gonochoristic females. This anatomical abnormality was rare before the 1960s, but has now been reported from many parts of the world, mostly from temperate regions, with the same prominent feature: association with boating or shipping activity. There is very little information available on such abnormality from developing countries. The importance of this research lies in showing that even a tropical area that is remote from major shipping routes, i.e., Ambon, Indonesia, showed the presence of the TBT biological indicator. Therefore, it appears to be at risk from the environmental impact of TBT-antifouling paints.

A reconnaissance survey was conducted in 1988 to determine whether neogastropod imposex occurred in Ambon Bay. As the abnormality was found there, follow-up field research was undertaken in 1989 to confirm the 1988 results. In both field studies, the sampling sites were selected based on their levels of marine activity; harbours and ferry docks were considered to be the Test Sites, while "clean" shores were treated as the Reference Sites. Specimens were collected as they were seen, in harbour areas by snorkeling divers and on shores by hand.

Species selection was based on specimen size, availability and accessibility in the field. Imposex was determined by the presence of a penis behind the right tentacle of females. Females were particularly distinguished from males by the presence of a sperm-ingesting gland. The diameter of a recurved penis was recorded as the penis length by using a 1 mm scale. Imposex incidence was

expressed by the Relative Frequency of Imposex (RFI), i.e. the relative ratio of imposex females to the total number of females. Imposex intensity was calculated by the Relative Penis Size (RPS) index, i.e. (mean length of female penis/mean length of male penis)<sup>3</sup> X 100%.

*Thais luteostoma*, an intertidal neogastropod mollusc, showed widespread imposex in Ambon Bay, Indonesia. All sites showed some imposex, but the higher levels of occurrence were recorded at the two harbours in the bay serving ocean-going traffic (RFI = 90-100% and RPS = 20 - 90%). A number of sites with low levels of incidence and intensity were spread around the harbours. An imported TBT-antifouling paint is available at a ship slipway, but is rarely used due to relatively high cost. Accordingly, the causative agent is suspected as TBT leaching from antifouling paints used by deep-sea vessels.

*Thais luteostoma* is the most suitable bioindicator for imposex study in Ambon Bay as a moderately large (2-3 cm shell length), moderately abundant and readily accessible species. It occurs from mid-tide into shallow water, on rocky shores, concrete and wooden pilings.

The investigation protocols developed allow for confirmation of the presence of neogastropod imposex in a laboratory without histological sectioning facilities, and also allow graphical intersite comparisons of imposex occurrence where small samples preclude rigorous statistical significance tests.

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and our baby boy, John

*A true scientist is not a reknown-seeking scientist,  
a good scientist is not an unethical scientist.*

## Chapter I

### INTRODUCTION

#### 1.1 Neogastropod Imposex

##### 1.1.1 The Importance of Imposex

Tributyltin (TBT) was introduced in antifouling paints in the 1960s (Evans, 1970). Its application is intended to effectively protect ships' hulls from biodeterioration by fouling organisms. However, once released into seawater, TBT becomes toxic to a wide spectrum of non-target organisms, ranging from plankton to fish. The toxic TBT levels can be as low as less than 1 nanogram per liter of tin (Cardwell and Sheldon, 1986). Edward D. Goldberg, Professor of Chemistry at Scripps Institution of Oceanography stated that TBT, the active ingredient of antifouling paint, is the most toxic compound man has introduced into the marine environment (in Champ and Lowenstein, 1983, p. 71).

Because TBT tends to accumulate in the surface microlayer of the water (i.e., < 1 mm thick) (Cleary and Stebbing, 1987), intertidal life becomes subject to this contaminant as the tide moves in and out. Impacts of a marine contaminant, such as TBT, usually become more significant if they are visible along the shores. Undesirable environmental effects of TBT were first noticed in the mid 1970s in Arcachon Bay, France, in the forms of the shell malformation (i.e., thickening and cavitation) and decreased larval settling of the Pacific Oyster, *Crassostrea gigas*. The same problems also caused failure in culture of the same species in Britain (Stebbing, 1985).

Neogastropod imposex, a masculinization syndrome, is another sublethal effect of TBT. Through a series of laboratory and field experiments in the 1980s,

particularly those conducted in Plymouth Marine Laboratory, England, TBT was confirmed as a cause of imposex in neogastropod molluscs. In *Nucella lapillus*, the abnormality could be induced at a much lower level of seawater TBT concentration (i.e.,  $< 0.5 \text{ ng Sn l}^{-1}$ , see Section 1.1.3) than that causing shell thickening in the European Flat Oyster, *Ostrea edulis* (i.e.,  $< 0.1 \text{ } \mu\text{g TBT l}^{-1}$  or  $< 40 \text{ ng Sn l}^{-1}$ , Thain and Waldock, 1986).

The Neogastropoda comprise many genera and widely distributed marine benthic species (Kay, 1979). Their size, low mobility and sensitivity to TBT make whelk imposex a suitable biological indicator that can provide an early warning of TBT contamination.

### 1.1.2 The Occurrence of Imposex

The term "imposex" was coined by Smith (1971) for "...superimposition of male characters on to unparasitized and parasitized females" in the species *Nassarius obsoletus* Say. The species normally has separate sexes (dioecious or gonochoristic). This is a well-established characteristic of most Prosobranch gastropods (Fretter and Graham, 1962; Webber, 1977), particularly the Order Neogastropoda (Ponder, 1973; Parker, 1982). This is in contrast to the other subclasses, Opisthobranchia and Pulmonata, which are virtually all functionally hermaphrodites (Coe, 1944; Parker, 1982), either simultaneously or sequentially. Smith considered imposex to be a pseudohermaphroditism; an abnormality with aberrant, non-functional, male characters in females, especially in the form of the penis and vas deferens.

Since the early 1960s, imposex has been recorded in various neogastropods, such as *Urosalpinx cinerea follyensis* Baker, *Eupleura caudata etterae* Baker (Griffith and Castagna, 1962), *Nucella lapillus* (L.) (Blaber, 1970), *Ocenebra erinacea* (L.)

(Poli *et al.*, 1971 in Gibbs *et al.*, 1990) and *Thais emarginata* (Deshayes) (Houston, 1971). It has now been recorded that females of at least 58 neogastropod species and subspecies of 33 genera are penis-bearing (see Appendix 1).

The course of imposex development varies considerably with species. Smith (1971) noted that imposex females of *Nassarius obsoletus* have one or more of the following characters: (1) a penis with a duct leading to (2) a vas deferens which passes back to the ventral channel of the capsule gland, and (3) convolution of the normally straight gonadal oviduct. The imposex penis of this species initially appears as a bump (Smith, 1971). Furthermore, Smith (1981a, b) found no evidence of reproductive incapacity caused by the sexual abnormality in this species.

Six stages in the development of imposex in *Nucella lapillus* were noted by Gibbs *et al.* (1987) (see Appendix 2). In this species, the imposex penis first appeared in the form of a small ridge (Gibbs *et al.*, 1987). Histological section of the imposex penis in the same species revealed the presence of a penial duct (Miller and Pondick, 1984). This was not confirmed in a previous histological section (Blaber, 1970). Accumulation of aborted capsules in the pallial oviduct eventually caused premature death (Gibbs and Bryan, 1986). In advanced development of imposex in this species, the prostate gland displaced the capsule gland dorsally or posteriorly. In a more severe condition, gametogenic changes take place in the gonad, in which oogenesis is supplanted by spermatogenesis (Gibbs *et al.*, 1988).

In *Ocenebra erinacea*, advanced imposex causes malformation of the oviduct, including absence of a genital papilla and a normal vulva, and deformation of a bursa copulatrix, leading to sterility (Gibbs *et al.*, 1990).

All four species of *Nucella* on the Pacific coast of North America show imposex (Short *et al.*, 1989; Bright and Ellis, 1990). In *N. lima* (Gmelin), the imposex penis develops first as a round bump, followed by the distal section of vas deferens commencing from the penial base, and then the proximal section of vas

deferens (Short *et al.*, 1989). No proliferation of vas deferens tissues over the genital papilla was observed in this species. The genital pore was blocked only with a severe condition of imposex. In *N. lamellosa* (Gmelin), the development of the vas deferens eventually occluded the genital pore causing sterility (Bright and Ellis, 1990). Imposex-caused sterility was not observed in *N. emarginata* and *N. canaliculata* (Duclos).

Six species of the widely distributed prosobranch gastropod, *Thais* [including two subspecies of *T. haemastoma* (L.) and the subject of this research, *T. luteostoma* (Holten)], have been reported as showing imposex. In *Thais clavigera* (Kuster), a penis was present in females throughout the year (Morton, *personal communication*, 1988). There was no observation made on the effects of imposex on the reproductive system in this species. Spence *et al.* (1990) and Stewart *et al.* (*in press*) observed the blockage of the vulva by the vas deferens in *T. haemastoma* and *T. orbita*, respectively. Accumulation of aborted capsules was also found in the capsule gland of *T. orbita*.

It can be seen that the severity and process of the effects of imposex on whelk populations varies according to species. Starting with penis and vas deferens growth, with no significant effects on the species reproductive system, the abnormality can eventually cause blockage of the genital pore resulting in species reproductive incapacity. This particularly affects the population ecology for those species lacking free-swimming larval stages because of the slow rate of recruitment from unaffected populations.

### 1.1.3 The Cause of Imposex

The appearance of a vestigial penis behind the right tentacle in females of some gonochoristic neogastropods has been reported as a normal, seasonal

phenomenon. A penis-like outgrowth was observed in spent females of *Nucella lapillus* (Blaber, 1970). Penis expression in females of *Ocenebra erinacea* (Feral, 1976 in Smith, 1981c) and *Nassarius obsoletus* (Smith, 1981c) were depressed before the breeding season and returned to the previous levels after the season. This is contrary to what was observed in males of *N. obsoletus* where the penis was reduced at the cessation of seasonal reproduction (Jenner and Chamberlain, 1955).

The occurrence of a penis behind the right tentacle of *Ocenebra erinacea* is stimulated by a morphogenetic factor (Feral, 1975 in Smith, 1981c). In prosobranch gastropods, the morphogenetic factor is produced by the right pedal ganglion. It is then accumulated in the base of the right tentacle, from where it diffuses into the adjacent morphogenetic territory (i.e., the area from the proximal end of the tentacle to the basal region of the penis) (Le Gall and Streiff, 1975). The morphogenetic factor is passive in gonochoristic females *O. erinacea*, but in males it is responsible for the growth and differentiation of secondary sexual characters (Streiff, 1966, 1967 and Streiff & Le Breton, 1970a, b as quoted in Smith, 1981c). It has been shown that tributyltin (TBT) can induce neuroendocrine activity so as to release abnormally a pedal morphogenetic factor responsible for the penis differentiation in females of *O. erinacea* (Feral and Le Gall, 1983).

Recent information shows that in *Nucella lapillus*, the female penis is not seasonally resorbed and is apparently an irreversible syndrome (i.e., once induced will not regress) (Bryan *et al.*, 1986; Gibbs *et al.*, 1987). Age appeared to be unrelated to the variation of female penis expression (Bryan *et al.*, 1986). In a comparative field experiment where two groups of *N. lapillus* exposed from hatching to a 'clean' site and a 'contaminated' site (TBT concentrations in water were  $< 0.5$  and  $9-19 \text{ ng Sn l}^{-1}$ , respectively), after 18 months Gibbs *et al.* (1988) found that the ratio of the mean penis length of females for the two sites was approximately 1:4. This result, plus other field transplantation experiments (Bryan *et al.*, 1986, 1987)

established the validity of a series of laboratory experiments on TBT-induced imposex (Gibbs *et al.*, 1988; Bryan *et al.*, 1986, 1987, 1988).

Neogastropod imposex has been shown to be associated with marine traffic in the U.K. (Bryan *et al.*, 1986, 1987; Davies *et al.*, 1987), the eastern Atlantic (Spence *et al.*, 1990), and the western Atlantic (Smith, 1981a, c); north Pacific (Short *et al.*, 1989), the eastern Pacific (Saavedra Alvarez and Ellis, 1990; Bright and Ellis, 1990), southeast Asia (Ellis and Pattisina, 1990), northeast Australia (Mitchell, 1989), and New Zealand (Smith and McVeagh, *in press*; Stewart *et al.*, *in press*). These records show an almost worldwide distribution: the north Atlantic, and north and south Pacific.

Some studies have been conducted on correlations between imposex and heavy metals. The studies were based on comparison of heavy metal levels in whole body tissues of *Nucella lapillus* and its degree of imposex.

Miller and Pondick (1984) statistically compared the heavy metal levels in the body tissues of *Nucella lapillus* from six sites on the New England coast, and correlated them with the frequency of imposex incidence (RFI) of each site. Of the six heavy metals examined {i.e., cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), tin (Sn) and zinc (Zn)}, Cd, Cu and Zn levels showed significant differences among sites, but Sn levels were below the detection limit:  $< 0.5 \mu\text{g g}^{-1}$  dry weight. The significant differences in the levels of Cd among sites did not correspond with the pattern of imposex incidence. Elevated levels of Cu were associated with imposex incidence, while the association of Zn and imposex was not as clear.

A further study was conducted by Bryan *et al.* (1986), in which the levels of heavy metals in the body tissues of the same species around southwest England were analyzed and plotted against the intensity of imposex (RPS). The results showed that high tissue levels of Cu, Zn, Pb (lead), Cd, Ag (silver), and As (arsenic) are not implicated in the induction of imposex, whereas low total concentrations of Sn

in the body tissues are very significantly correlated to the degree of imposex in *Nucella lapillus*. Much of this Sn is hexane-extractable, including TBT and DBT (dibutyltin) fractions. Furthermore, in a transplantation experiment of *Nucella lapillus* from a 'clean' to a 'contaminated' site, Bryan *et al.* (1986) found a marked increase in imposex intensities of the transplants from 0.6% to about 60% and a corresponding increase of TBT fraction (i.e., 60-70% of the hexane-extractable Sn) in their tissues. Although these results were not totally conclusive, Bryan *et al.* confirmed them later through laboratory experiments (see Appendices 3 and 4).

The pattern of coincidence between high degree of imposex occurrence and proximity to harbours and marinas suggests that the causative agent of whelk imposex is almost certainly associated with shipping or boating activity (Smith, 1981a, b; Miller and Pondick, 1984; Bryan *et al.*, 1986).

Laboratory experiments on the induction of imposex in *Nucella lapillus* by various marina-associated materials, such as chemical toilet disinfectant, spray detergent and leaded gasoline (Smith, 1981a), yacht enamel (Bryan *et al.*, 1987) and antifouling paints (see Appendix 3) revealed that only TBT-based antifoulants were related to the occurrence of imposex.

Further experiments with organotin compounds only (see Appendix 4) showed that TBT is the most effective causative agent of imposex (Bryan *et al.*, 1988); two other organotins, tetrabutyltin (TTBT) and tripropyltin (TPrT), also caused imposex but to a much lesser extent (Bryan *et al.*, 1988).

The biocide tributyltin (TBT) has been confirmed as a causative agent for imposex in *Nucella lapillus* (Bryan *et al.*, 1987, 1988; Gibbs *et al.*, 1988), *Ocenebra erinacea* (Feral and Gall, 1982 in Bryan *et al.*, 1987) and *Nassarius obsoletus* (Smith, 1981b). The imposed male sex characters in these three species are initiated at different levels of seawater TBT concentrations:  $< 0.5 \text{ ng Sn l}^{-1}$  for *Nucella*

*lapillus* (Gibbs *et al.*, 1988), about 1.0 ng Sn l<sup>-1</sup> for *Ocenebra erinacea* (Gibbs *et al.*, 1990), and about 2.0 ng Sn l<sup>-1</sup> for *Nassarius obsoletus* (Bryan *et al.*, 1989a).

The biocidal activity of organotin compounds was first recognized in 1943 (Tisdale, 1943 in Evans, 1970). However, it was not until about 20 years later (i.e., mid 1960s) that they were widely used in marine antifouling paints (Evans, 1970). Such paints were introduced to both Scottish and English yachtsmen in 1969/1970 (Bailey and Davies, 1989). In Victoria, Canada, the first readily available record of their routine use was in 1973 at the naval dockyard (Kirk, 1973). In New Zealand, they were not in use until after 1971 (Smith and McVeagh, *in press*).

Prior to the introduction of TBT antifoulants in the 1960s, most references to the reproductive systems of neogastropod molluscs gave no signs that the imposex abnormality occurred. Major anatomical studies in the early 1940s on the sexual system of gastropod molluscs, e.g., Fretter (1941) and Coe (1944), did not report such abnormalities. Fretter's work (1941) on the genital ducts of some neogastropods is particularly interesting since it included three species, i.e., *Ocenebra erinacea*, *Nucella lapillus* and *Nassarius reticulatus*, which now show frequent and intense imposex. In addition, at least four sets of preserved museum specimens and one set of historical records showed no sign of imposex in areas now with up to 100% incidence: four and five female of *Ocenebra erinacea* collected in west France (1882) and in southwest England (1949) (Gibbs *et al.*, 1990); four female *Nucella lapillus* collected in southwest England (1936) (Moore, 1936 in Bryan *et al.*, 1986); six and eight female *N. lamellosa* collected in eastern (July, 1959) and western Vancouver Island (August 1961), respectively (Bright and Ellis, 1990); six female *N. emarginata* collected in Washington, U.S.A. (summer 1958) (Bright and Ellis, 1990); and 498 *Lepsiella scobina* Quoy & Gaimard collected in northern New Zealand (1970-71) (Smith and McVeagh, *in press*).

Based on the results of laboratory and field experiments, supported by field observations and historical museum data, it is clear that the biocide tributyltin (TBT) induces imposex, apparently indirectly through its effects on the neuroendocrine system in gonochoristic neogastropods.

The reversibility of the penis expression (i.e., seasonal penis regression) has been reported in *Nucella lapillus* (Blaber, 1970), *Ocenebra erinacea* (Feral, 1976 in Smith, 1981c) and *Nassarius obsoletus* (Smith, 1981c). Later, it was observed in *Nucella lapillus* that the penis expression is irreversible (Bryan *et al.*, 1986; Gibbs *et al.*, 1987). This contradiction needs not complicate the use of neogastropod imposex to indicate the presence of TBT contaminant. If imposex, reversible or not, shows a pattern of distribution associated with marine activity, TBT should be suspected as a contaminant.

The experiment of Feral and Le Gall (1983) showed that TBT can be hypothesized to affect the hormonal balance controlling the appearance of the penis in normal gonochoristic females. The TBT-induced release of the morphogenetic factor in the affected females means they have the same two factors controlling penis differentiation (i.e., the morphogenetic and retrogressive factors) that the gonochoristic males have. The retrogressive factor, which is released by the pleural ganglia, is already present and active in normal females (Feral and Le Gall, 1983). Its function is to control the penis regression (Le Gall and Streiff, 1975). The penis reversibility suggests that this factor is still active in the TBT-affected females. Its action in the breeding season is probably induced by ovarian maturation. However, in a life-long exposure to TBT contamination, the retrogressive factor is probably inactivated in some way. If this is so, then the imposex irreversibility is a chronic effect of TBT contamination. The fact that the imposex irreversibility is only confirmed so far for *Nucella lapillus* suggests that the phenomenon is species-specific.

Contradictory results between Miller and Pondick (1984) and Bryan *et al.* (1986) on the association between the tissue levels of copper (Cu) and imposex showed that the results of the statistical analyses of biological data, e.g., Miller and Pondick (1984), should be carefully interpreted. If possible, statistical significance should be confirmed through biological methods, e.g., experimental induction of imposex. If such studies are to be conducted in future, they should include chemical analyses of the heavy metals in seawater in the forms that are biologically available to the whelks.

This collated information does not mean that TBT is the sole environmental cause of neogastropod imposex. There may be other causes, but there is currently no information indicating what they might be.

#### 1.1.4 Imposex as a TBT Bioindicator

The findings that tetrabutyltin (TTBT) and tripropyltin (TPrT) have some capacity to induce imposex do not diminish the value of imposex as an indicator of TBT contamination since the effect of TTBT may depend on its degradation to TBT and TPrT is rarely used in antifouling paints (Bryan *et al.*, 1988). TBT-based antifouling paints have been in a widespread use since the early 1970s (Champ and Lowenstein, 1983).

Reviews of TBT toxicity in aquatic life by Thompson *et al.* (1985), Champ (1986) and Cardwell and Sheldon (1986) show that TBT toxic concentrations in water for most non-target (i.e., non-fouling) organisms are in the microgram per liter range. The lowest active concentration recorded in seawater was  $0.006 \mu\text{g l}^{-1}$  of TBT or  $2.4 \text{ ng l}^{-1}$  of tin (96 hours-EC<sub>50</sub>) affecting larvae of the bivalve *Mercenaria mercenaria* (Becerra-Huencho, 1984 in Cardwell and Sheldon, 1986). At these low levels, whelk imposex proves to be the most sensitive bioindicator of TBT

impact yet discovered since it is initiated at concentrations less than  $0.5 \text{ ng l}^{-1}$  of tin. In addition, whelks are also easy to collect and the deleterious effect of TBT on them is not acutely lethal.

Several indicator variables are used to assess the impact of TBT contamination. Blaber (1970) used the percentage of affected females (penis-bearing females) as a measure of the degree of imposex. This frequency index is the simplest and most sensitive measure of imposex (Ellis and Pattisina, 1990). However, in highly contaminated areas, where all females are affected (i.e., imposex incidence = 100%), other imposex indices are needed to measure the intensity of the effect. These measures have been developed from two notable imposex characters, i.e., the penis and the vas deferens.

Penis size was found to be the easiest and most reliable parameter to measure (Smith, 1981a; Gibbs *et al.*, 1987). The cube of penis length of both sexes was shown to be proportional to its wet weight (volume) (Bryan *et al.*, 1986). This is as expected for the general length-weight relationship:  $W = aL^b$ , where  $W$  is weight,  $L$  is length and  $b$  is a constant usually close to 3 (Gulland, 1983). Therefore, a simple numerical index, the Relative Penis Size (RPS) expressed as  $(\text{mean female penis length})^3 / (\text{mean male penis length})^3 \times 100\%$ , was developed to measure the degree of imposex intensity (Bryan *et al.*, 1986). Since the RPS index does not indicate the reproductive competency of the affected females, Gibbs *et al.* (1987) developed a six-stage vas deferens sequence (VDS) (see Appendix 2), and calculated the mean of the stages as the VDS index. These biological indices can provide quick and inexpensive means to quantify the degree of imposex in order to assess the risks of TBT contamination.

As shown in Appendix 1, many neogastropod molluscs are potentially good indicators of TBT contamination. If they are to be used as biological indicators, research on them needs to demonstrate patterns of concentrated distribution which

suggest point sources of contamination, rather than occurring at random. If patterns of such 'hotspots' appear, for example, near harbours and marinas, then point sources of TBT contamination are indicated.

Neogastropod imposex is a valuable bioindicator for early warning of TBT contamination since it is simple, quick and inexpensive. After a preliminary survey of imposex, suspected sources of TBT contamination can then be subjected to the more time-consuming and expensive chemical analyses to confirm whether or not the contaminant is present at environmentally significant levels.

#### 1.1.5 Definitions of Technical Imposex Terms

Some terms used here are defined as follows:

- Imposex incidence (incidence of imposex) is the frequency of the abnormality (i.e., an imposex penis as used in this research) on individual females. It is expressed by the Relative Frequency of Imposex (RFI) or imposex frequency index.
- Imposex intensity (intensity of imposex) is the degree of expression of the abnormality in forms of penis length. It is expressed by the index Relative Penis Size (RPS). Potential and actual intensities of imposex are indicated by Population and Incidence RPS indices, respectively (see Chapter II). In practice, however, all the values of the penis length or indices calculated based on the penis length (i.e., RPS indices) in this research are only approximates due to the recurved form of the main penial body.
- Imposex occurrence (occurrence of imposex) is a collective term for both imposex incidence and intensity.

## 1.2

**Objectives**

The main objective of this research was to determine whether neogastropod imposex occurred in a tropical area that was remote from major shipping routes. If imposex did occur, did it show patterns of distribution indicating that tributyltin antifouling paints were the cause? The importance of the research lies in showing that even remote tropical areas could be at risk from the known environmental impacts of these paints, if those areas are regularly visited by ocean-going vessels.

Opportunities for field and laboratory work were provided by Pattimura University in Ambon, Indonesia.

Once the occurrence of imposex was established, the following objectives were then set:

- i. Determine whether the usual causative contaminant, tributyltin, was in use in the area, and under what circumstances.
- ii. Determine which species showed imposex, which were most suitable as indicator species for tributyltin contamination surveys and for what reasons, and develop a protocol for species selection in new, taxonomically little-explored areas.
- iii. Check existing measures of imposex as quantitative indices, develop a numerical protocol for measuring imposex, and develop a protocol for comparing measures from different locations or at different times.
- iv. Establish the level of imposex at the investigated site in form suitable for intersite comparisons.

Note that this research does not investigate TBT levels by chemical analysis. Funds were not available. The results of this research, however, do show that such analyses are now needed, and demonstrate the locations for appropriate sampling.

The initial field survey to establish the occurrence of imposex, determine the species showing it, collect specimens for laboratory work, make preliminary analyses, and draw preliminary conclusions was undertaken from May until August 1988 (Chapter 2).

A second short field season was undertaken in May 1989. This was to collect specimens from more sites to test the hypothesis that imposex occurrence in Ambon Bay was likely to be derived from TBT antifouling paints, as indicated by imposex "hotspots" associated with marine traffic activity. The data from 1988 and 1989 were used to develop numerical and graphical protocols for comparisons of imposex occurrence (Chapters 2 & 3).

The second field season was followed in 1989 and 1990 by microscopy of body tissue samples to develop improved gender determining procedures. These results are reported in Chapter 4.

Chapter 5 presents a discussion of the various factors which need consideration in the application of the imposex protocol in new areas, summarizes conclusions based on the objectives stated before, and gives recommendations for future direction in imposex investigation in new areas, particularly Ambon and other tropical areas.

### 1.3

#### **The Study Area: Ambon Bay**

##### **1.3.1 Geography and Climate**

Ambon Island is located at longitude  $127^{\circ}52'$ -  $128^{\circ}19'$  E and latitude  $3^{\circ}30'$ - $3^{\circ}47'$  S, approximately at the center of the administrative region of Maluku (Moluccas) Province in eastern Indonesia (Figure 1.1). The island consists of two

peninsulas, Leihitu and Leitimur enclosing Ambon Bay (see the Inset in Figure 1.1). It is the largest bay on the island, and supports the most vessel traffic.

The climate is dominated by two annual seasonal changes, the East and West Monsoons. In the East Monsoon (Wet Season) during April-September, the southeasterly wind brings rain over the island (Figure 1.2). The Dry Season occurs during the West Monsoon from October to March.

Ambon Bay has inner and outer parts separated by a narrow 14 m depth Galala-Rumahtiga Sill (Wenno, 1986 in Hamzah and Wenno, 1987) (see the Inset in Figure 1.1). The inner bay is sheltered and relatively calm through the year. The outer bay is exposed to the Banda Sea. The strongest waves hit the steep southeastern coast during the West Monsoon, and the less steep northwestern coast in the East Monsoon (Hermanto, 1987).

Table 1.1 Seawater temperature ( $^{\circ}\text{C}$ ) of Ambon Bay<sup>1)</sup> and Banda Sea<sup>2)</sup>  
[Source: Tarigan and Sapulete, 1987]

Location	Depth <sup>3)</sup>	West Monsoon	East Monsoon
		Range (mean, n=8)	Range (mean, n=8)
Ambon Bay (inner)	1	29.2-30.7 (29.7)	26.5-27.5 (26.9)
	2	27.6-29.2 (28.3)	24.7-27.4 (25.7)
Ambon Bay (outer)	1	28.2-29.7 (29.1)	25.1-26.9 (26.0)
	2	28.0-29.3 (28.5)	24.6-27.2 (25.7)
Banda Sea	3	27.0-28.0 (-)	25.5-27.0 (-)

Legend: 1) Annual mean temperature from 1973-1980;  
2) No time record;  
3) 1 = 0.5 m; 2 = 1 m above seabed; 3 = 0-25 m.

Based on the seawater temperature data shown in Table 1.1, it appears that good exchange of water masses between Ambon Bay and the Banda Sea occurs during the East Monsoon, as indicated by the relatively homogeneous temperature

Figure 1.1 **Ambon Island in the Central Indo-Pacific Region** [*base map is the courtesy of Hinton, 1972; Inset: Ambon Island showing the position of the study site, Ambon Bay*]

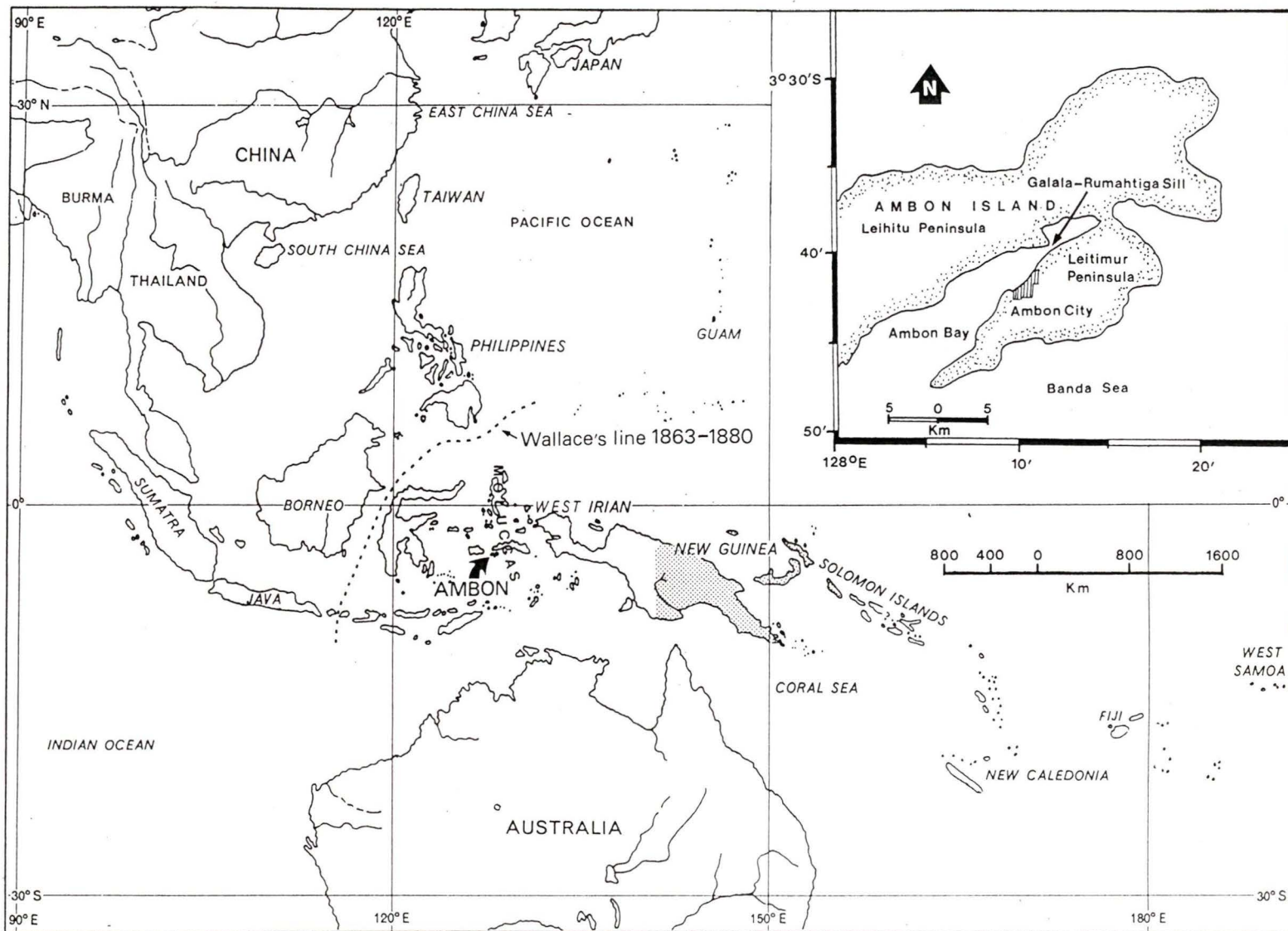
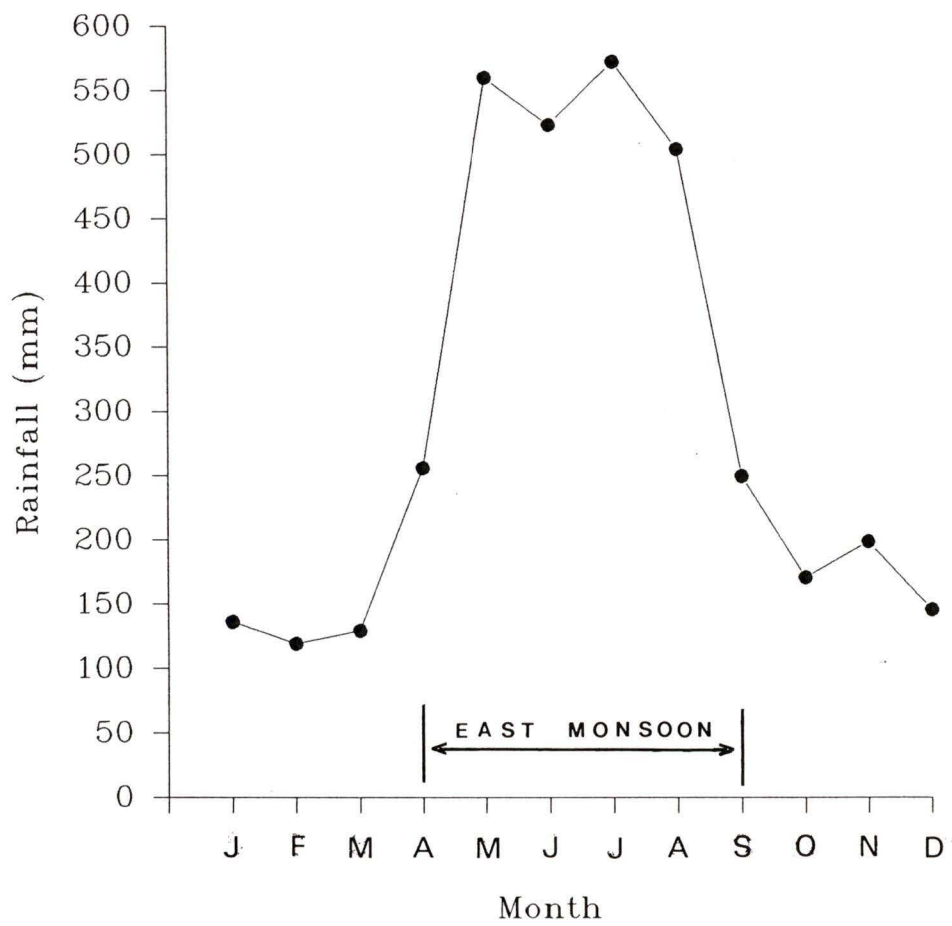


Figure 1.2 **Monthly rainfall curve for Ambon** [Source: *Sukanto, M., 1969, p. 217 - average monthly rainfall data for Ambon, 1931-1960*]



distribution between them. This can be explained by the position and shape of the mouth of Ambon Bay, which is open to the Banda Sea. Therefore, it receives the Banda Sea's water masses driven by the southeasterly wind during the East Monsoon. Lower average temperatures recorded for the outer bay compared to the records for the inner bay indicated that the outer bay is better flushed, particularly in the East Monsoon. Wenno (1986 in Hamzah and Wenno, 1987) found that the exchange of water masses between the two parts of the bay is slightly restricted by the Galala-Rumahtiga Sill.

### 1.3.2 Biogeography

Ambon Island is in the Central Indo-West Pacific. This is the area bordered by the Philippines, Malaysia and Papua New Guinea. It is considered as the biogeographic center of the Indo-West Pacific Region (Briggs, 1974).

Ambon Island is located to the east of the Wallace's Line (see Figure 1.1), the boundary between the Oriental and Australian Realms (Whitmore, 1987). It is, therefore, expected that its faunal relationships in general will be more Australian than Asiatic.

Calculations based on the data presented in Wells (1990), show that there are 426 species of shallow-water marine neogastropods known in Indonesia (excluding western New Guinea: Irian Jaya). Of this number, 357 species (83.8%) and 347 species (81.5%) were reported from New Guinea (including the western half) and northern (i.e., tropical) Australia, respectively.

### 1.3.3 The Selected Study Sites

In total, eight sites were sampled. During the preliminary field season in 1988, several sites were examined, and samples of neogastropod molluscs were collected from six. During the second field season in 1989, a further two sites were added. The sampling sites, therefore, span both northwest and southeast shores of the bay (Figure 1.3). The photographs of each sampling site are shown in Figure 1.4.

Amahusu is a small village outside Ambon City. Its shore habitat is dominated by sand and rocks. Local fishermen use small canoes for fishing.

Gudang Arang Harbour was one of the main harbours of Ambon. It served inter-island vessels. Concrete pilings with some wooden struts support the docks. It has not been formally used for approximately 10-15 years.

Yos Soedarso Harbour is the main harbour in Ambon City, serving transportation within Maluku and between the provinces. The harbour's pilings are mainly concrete.

Slamet Riyadi Harbour is about 500 m to the northeast of Yos Soedarso. It mainly serves wooden ships used for local travel. Most of its pilings are wooden with some concrete structures.

Galala Tuna Harbour was specially built for tuna-fishing vessels, and contains a fish-packing plant. Foreign vessels, mostly Japanese, visit this harbour. The pilings are wooden.

Galala Ferry Dock is located about 500 m to the northeast of Galala Tuna Harbour. There is bare rock habitat on both sides of the port.

Poka Ferry Dock serves the ferry on the northwest side of the bay. There are muddy shores dominated by mangrove trees on both sides of the port. There is a damaged wooden ship to the southwest on which gastropod molluscs occur.

Poka Shore is a sandy-mangrove shore. At about mid-tide level there is half-buried wooden ship that supports gastropods. They also occur on the mangrove trunks and aerial roots.

Figure 1.3 **Sampling sites in Ambon Bay** [closed circle = *shore*; triangle = *high-sea harbour*; open circle = *wooden-boat harbour*; square = *ferry dock*; 1 = *Amahusu (1989)*; 2 = *Gudang Arang (1988)*; 3 = *Yos Soedarso (1988)*; 4 = *Slamet Riyadi (1988)*; 5 = *Galala Tuna (1988/89)*; 6 = *Galala Ferry (1989)*; 7 = *Poka Ferry (1988/89)*; 8 = *Poka (1988)*]

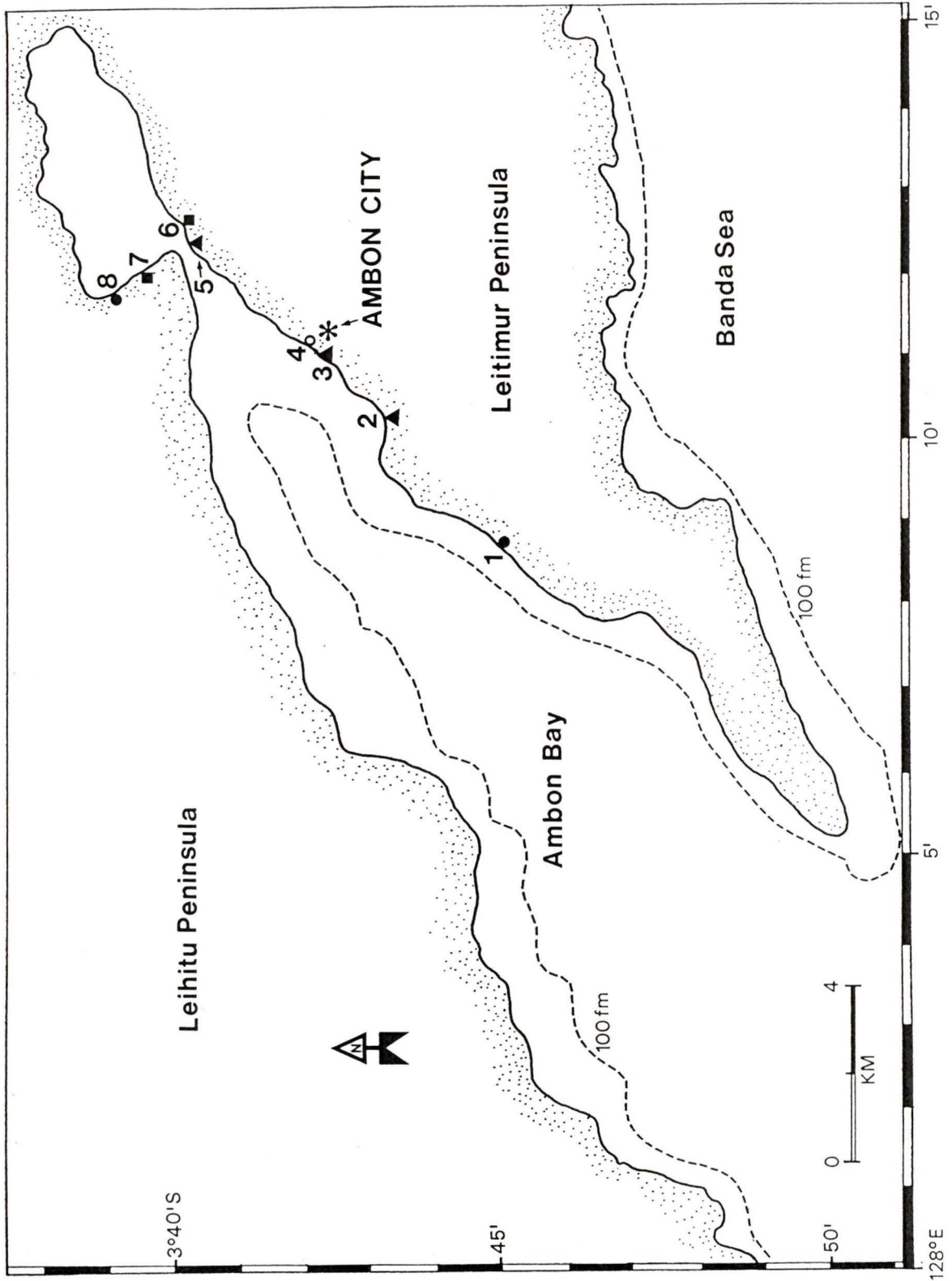
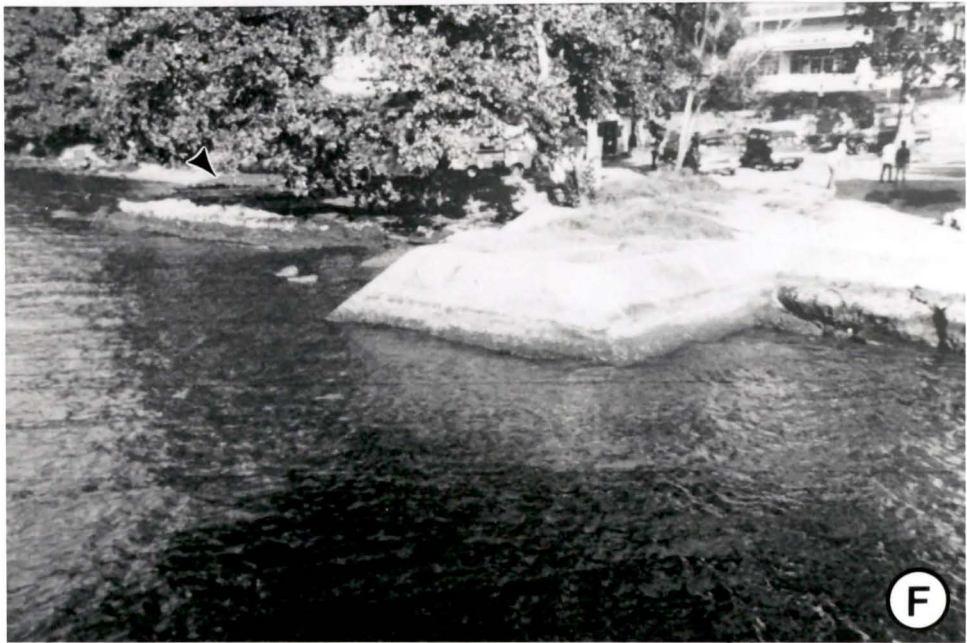
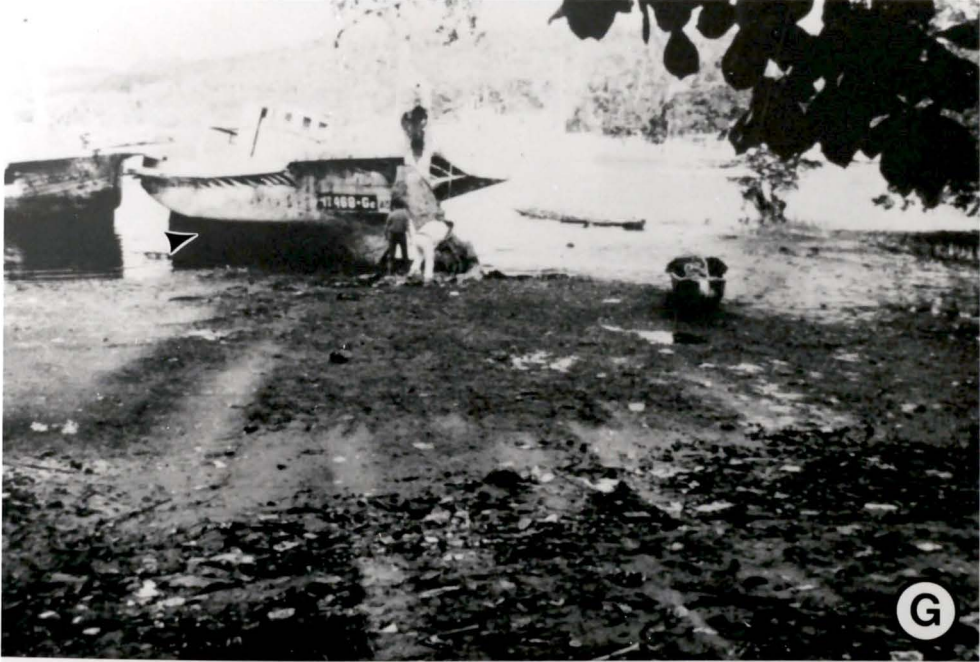


Figure 1.4 **Photographs of the sampling sites** [A = *Amahusu Shore (the sampled habitat is not shown in the picture)*; B = *Gudang Arang Harbour*; C = *Yos Soedarso Harbour*; D = *Slamet Riyadi Harbour*; E = *Galala Tuna Harbour*; F = *Galala Ferry Dock*; G = *Poka Ferry Dock*; H = *Poka Shore (the sampled habitat is not shown in the picture)*; arrowheads point to the sampled habitats; photographs taken from colour slides by D.V. Ellis]









## Chapter II

### RECONNAISSANCE SURVEY 1988 AT AMBON BAY

#### 2.1 Introduction

The first objective of the reconnaissance survey in 1988 was to determine whether neogastropod imposex occurred in Ambon. If this was confirmed, the next objective was to determine whether TBT-based antifouling paints were in use in the area. Finally, the third objective was to demonstrate whether there were any patterns of imposex occurrence by species and by location.

#### 2.2 Materials and Methods

Field work in 1988 was conducted from May 23 to August 30. Biological surveys were conducted on dates, times relative to tides, and at locations shown in Table 2.1 and Figure 1.3. Surveys of marine paint use were conducted on the dates and in the form shown in Table 2.2 and Appendix 5.

The balance of the time was spent in making preparations for the surveys, examining specimens using the facilities of the Laboratorium Kultivasi Universitas Pattimura (Aquaculture Laboratory of the Pattimura University) in Poka, Ambon, and collating data. Several unsuitable sites were eliminated by field inspections.

##### 2.2.1 Field Collections

At the four harbours, due to difficulty in organizing access from land, snorkel divers were used to collect specimens from hard surfaces of pilings. Diving collections were timed to be close to slack water of highest or lowest

tides. There were no attempts to randomize samples in these underwater collections. Specimens were collected as they were seen by the divers. Collections at beach sites were by hand, and shovel and screen (5 mm mesh) and were done at available daylight low tides. Specimens were kept in plastic bags with seawater, and returned to the laboratory for identification and examination.

Table 2.1 Timing of biological survey, 1988 [see also Figure 1.3]

Date, 1988	Site <sup>1)</sup>	Visited time	High tide		Low tide		Type of collection
			Time	H <sup>2)</sup>	Time	H <sup>2)</sup>	
July 2	GTH	14:15- 16:45	04:00 15:00	1.5 2.0	08:00 22:00	1.0 0.0	Diving
July 11	GAH	09:30- 12:45	01:00 10:00	1.3 1.9	03:00 18:00	1.2 0.1	Diving
July 16	YSH	14:00- 17:30	04:00 15:00	1.5 1.8	09:00 22:00	1.0 0.1	Diving
July 23	PS	08:00- 11:00	07:00	1.9	01:00 14:00	0.9 0.5	Hand
Aug. 2	PFD	09:45- 12:30	05:00 16:00	1.9 1.7	11:00 22:00	0.4 0.4	Hand; Digging
Aug. 5	SRH	12:30- 15:30	06:00 19:00	2.0 1.2	13:00 23:00	0.3 0.9	Diving

Legend:

1) GTH = Galala Tuna Harbour; GAH = Gudang Arang Harbour; YSH = Yos Soedarso Harbour; PS = Poka Shore; PFD = Poka Ferry Dock; SRH = Slamet Riyadi Harbour.

2) Height in meters.

Surveys of marine paint use were conducted at hardware stores and a ship slipway. Information was obtained by examination of paint labels and by informal questioning (see Table 2.2 and Appendix 5).

Table 2.2 Timing of marine paint use survey

Date, 1988	Name	<u>Location</u>	Type	Method used
Aug. 10	Guna Rakyat, Sama Lagi, Angin Timur, Rekayasa,	Hardware stores		Examined paint labels and inter- viewed shopkeepers
Aug. 30	Perum Perikani Galala,	Slipway		Interviewed the foreman and obtained brochures

### 2.2.2 Species Identification and Taxonomy

After each field survey, specimens were assembled as apparent species. Shell characters were matched to short descriptions and figures in the available literature, and later to illustrations in Wilson and Gillett (1971), Hinton (1972) and Cernohorsky (1971, 1972). Body tissue characters needed examination to identify specimens with damaged shells. Identifications were primarily to genera. Some help was obtained from Miss Prully Uneputty (a student of the Fisheries Faculty, Pattimura University), particularly with a specimen identified as *Mancinella* sp.

For verification of the preliminary identification of the species showing imposex, some shells were taken to the Department of Zoology of the University of Hong Kong for a comparative study with the neogastropod collection of Dr. Brian Morton. At his suggestion, specimens were sent to Dr. John Taylor at the British Museum (Natural History) in England. Subsequently, they were examined by Dr. Jan Sigurdsson at the Zoology Department, National University of Singapore in Singapore and Dr. Silvard P. Kool at the Museum of Comparative Zoology, Harvard University in U.S.A.

### 2.2.3 Imposex Measures and Data Analyses

The procedures of Gibbs *et al.* (1987) were followed. Each specimen shell was cracked in a bench-top vise, the body removed and examined under a binocular microscope to determine the sex. Females were determined by the presence of any of the sperm-ingesting gland, the capsule gland or the genital papilla. The imposex penis is an outgrowth behind the right tentacle, the location of the male penis (Figure 2.1). A vas deferens, if present in the female, extends between the penis and the genital papilla.

Routine measures included length, width and height of shell (Figure 2.2), penis length and coded measure of the vas deferens sequence. Penis length was measured by a water resistant 1 mm scale placed under the penis (Figure 2.3). Since the main body of the penis in the selected species is recurved with a coiled flagellum, the length was measured as the diameter of the recurved body. Blank worksheets used for recording imposex are provided as Appendix 6.

The level of imposex can be expressed by the Relative Penis Size (RPS), the index developed by Bryan *et al.* (1986):

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

A female lacking any measurable penis outgrowth is included as a zero.

In practice, the index is more efficiently calculated as:

$$\left[ \frac{\text{mean length of female penis}}{\text{mean length of male penis}} \right]^3 \times 100\%$$

Figure 2.1 **Male and female organs in a neogastropod snail, *Nucella lapillus***  
[Figures are redrawn from Gibbs *et al.*, 1987, p. 509; f = foot; vd = vas deferens; rt = right tentacle; p = penis; me = mantle edge; hg = hypobranchial gland; rg = rectal gland; pr = prostate; k = kidney; t = testis; dg = digestive gland; cm = columella muscle; o = operculum; cg = capsule gland; sg = sperm-ingesting gland; ag = albumen gland; ov = ovary]

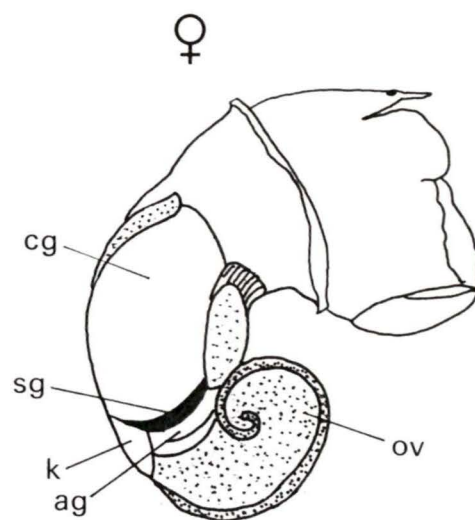
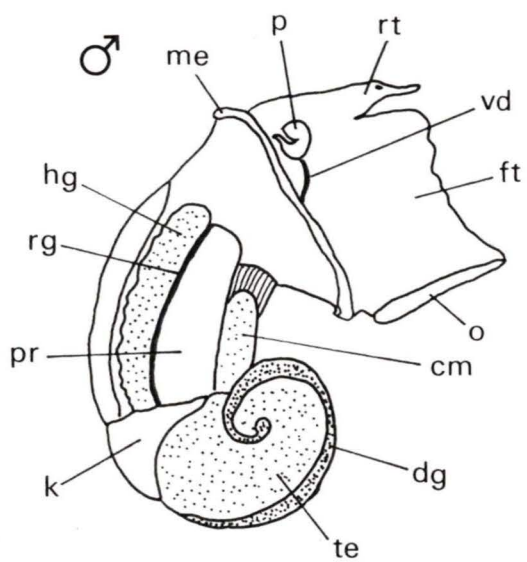


Figure 2.2 **Shell length, width and height measures** [*l* = length; *w* = width; *h* = height]

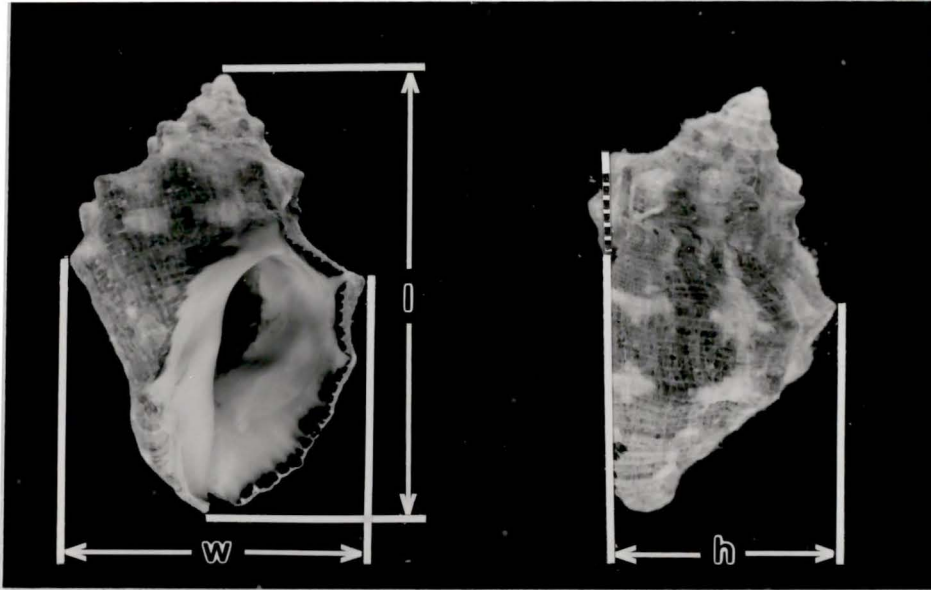
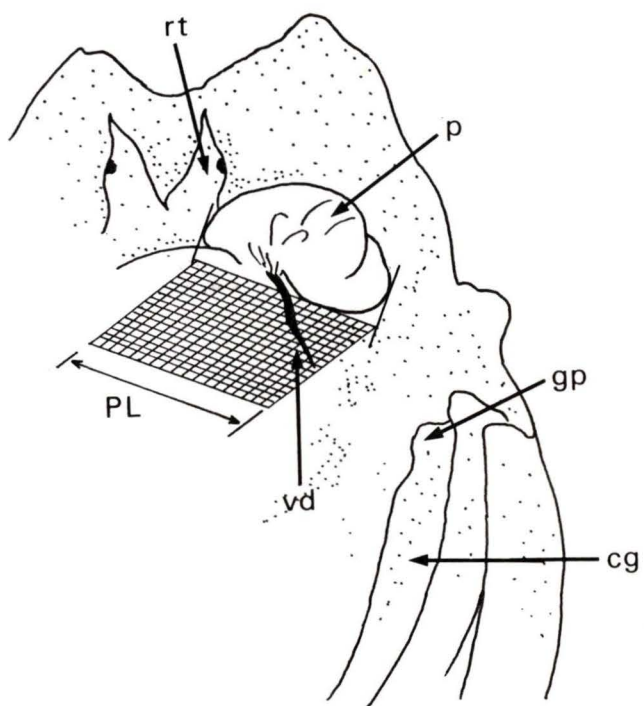


Figure 2.3 **Penis length measure** [PL = *penis length*; rt = *right tentacle*; p = *penis*; vd = *vas deferens*; gp = *genital pore*; cg = *capsule gland*]



Using the last expression, three RPS measures were calculated by modifying the number of females standardized against all the male population. The Population RPS ( $RPS_{pop}$ ) was calculated as of Bryan *et al.* (1986), i.e., from all females including those without imposex (penis length = 0 mm). The Incidence RPS ( $RPS_{inc}$ ) was calculated from only those females showing imposex. The Individual RPS ( $RPS_{ind}$ ), which was specially developed for graphical intersite comparison of imposex occurrence, was calculated as:

$$\left[ \frac{\text{individual female penis length}}{\text{mean male penis length}} \right]^3 \times 100\%$$

Harvard Graphics version 2.12 (Software Publishing Corporation, 1988) was used to generate three dimensional frequency bar diagrams for the incrementally-ordered Individual RPS values.

In addition, the Relative Frequency of Imposex (RFI), i.e., the relative (percentage) ratio of imposex female to the total number of females, also provided a simple index. Vas deferens sequence stages were determined, and the VDS index was calculated as the mean of the stages (Gibbs *et al.*, 1987).

Some sexually undeterminable specimens were preserved in buffered formalin (Humason, 1979) and taken to the University of Victoria for gonad examination.

Female penis length measures were checked for their normality and homoscedasticity (homogeneity of variance) (Sokal and Rohlf, 1981). The SYSTAT package of programs (Wilkinson, 1988) was used, allowing the Lilliefors Test for normality and Bartlett Test for homoscedasticity (Sokal and Rohlf, 1981). Statistical significance is weighed by the probability value (P-value); the smaller the P-value, the stronger is the evidence against the null hypothesis ( $H_0$ ) provided by the data (Moore and McCabe, 1989). The criteria used to draw conclusions are based on the

5% level of significance. In addition, Probability Plots are generated by the same statistical program. A straight line in the Plot indicates a normal distribution (Sokal and Rohlf, 1981). The Plot is particularly intended for making a decisive conclusion on the normality if the P-value is only slightly different from the level of significance.

As the results violated the underlying general assumptions of parametric statistical tests (see Section 2.3.3), no tests of significance were undertaken. Small sample sizes precluded the use of Chi-square test for independence of imposex on site since more than 20% of the cells have expected frequency less than 5.0 (Cox, 1990). Accordingly, site comparisons of imposex occurrence were made by tabular and graphical presentations only.

#### 2.2.4 Tributyltin Use Survey

The steps of the survey are outlined in Figure 2.4 with the timing shown in Table 2.2 and typical questions in Appendix 5.

The foreman of Perum Perikani Slipway in Galala was interviewed about the antifouling paints usually used at the slipway for painting ships.

### 2.3

### Results

#### 2.3.1 Species Identifications and Distribution

Five species were collected from the six sites (see Table 2.3). The most widely distributed of these (see Figure 2.5) was matched to illustrations of *Thais kieneri* (Deshayes, 1844) (Shaw, 1980). It was later identified by Dr. J. D. Taylor as *Thais clavigera* (Kuster, 1858), and subsequently confirmed

Figure 2.4 **Flow diagram of the search for antifouling paint information in hardware stores**

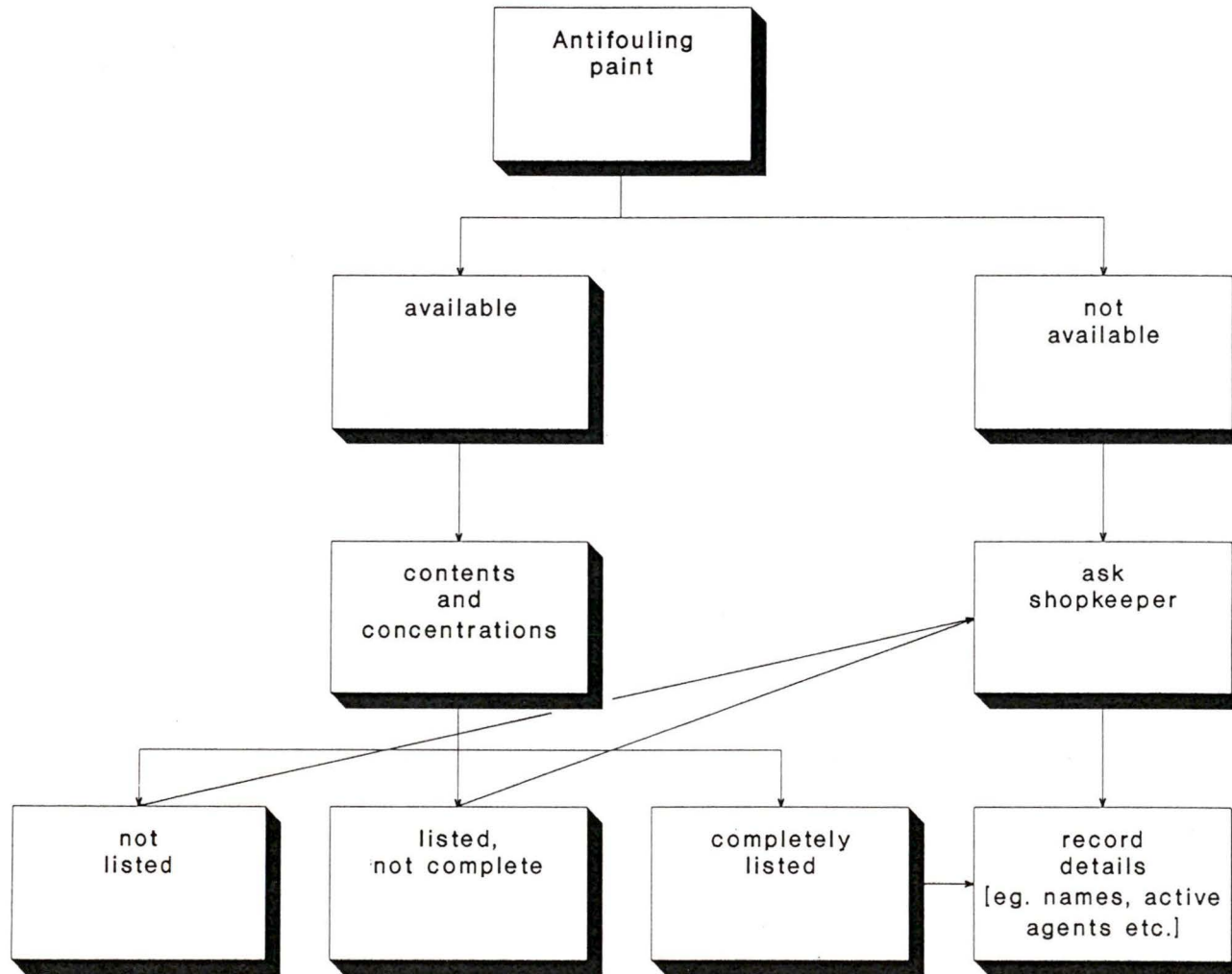
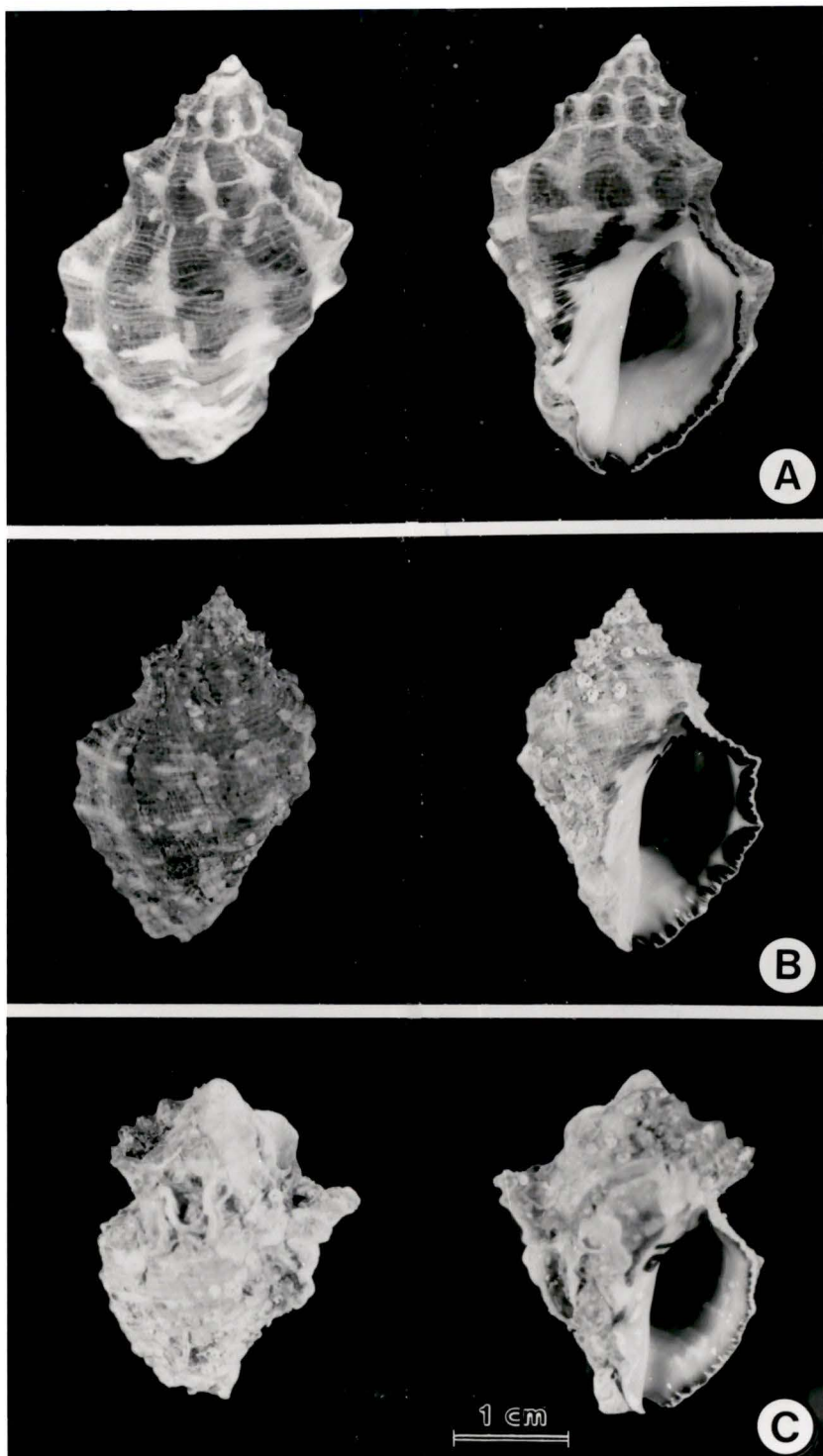


Figure 2.5 **Specimens of *Thais luteostoma* showing shell variability** [A = relatively clean; B = moderately fouled; C = heavily fouled]



as *T. luteostoma* (Holten, 1803) by Drs. J. Sigurdsson and S. P. Kool (Table 2.4), with a note that *T. kieneri* is a junior synonym. This species was selected as a bioindicator and it provided all the imposex data necessary to meet the research objectives.

Table 2.3 Distribution and abundance\* of the collected species at the sampling sites, 1988

Date 1988	Site	TL	N	Species		Mo
				Ma	C	
Aug.2	Poka Ferry Dock	19	+	-	-	+
Jul.23	Poka Shore	24	-	-	-	+
Jul.2	Galala Tuna Harbour	28	-	+	+	-
Aug.5	Slamet Riyadi Harbour	35	-	-	-	-
Jul.16	Yos Soedarso Harbour	26	-	-	-	-
Jul.11	Gudang Arang Harbour	8	-	+	-	-

Legend : \* = only counted for the selected species;  
 TL= *Thais luteostoma*; N= *Nassarius* sp.; Ma= *Mancinella* sp.;  
 C = *Chicoreus* sp.; Mo= *Morula* sp.  
 + = present; - = absent;

Notes on the species' substrates:

*Thais luteostoma*: wood, wooden and concrete pilings;  
*Nassarius* sp. : muds (30-40 cm depth);  
*Mancinella* sp. : wooden and concrete pilings;  
*Chicoreus* sp. : wooden pilings;  
*Morula* sp. : mangrove trunks and roots.

The other species were identified as *Nassarius* sp., *Mancinella* sp., *Chicoreus* sp. and *Morula* sp. Of the five species collected, only *Thais luteostoma* was widespread at the accessible sites.

Table 2.4 Identifications of the species showing imposex

Taxonomist	Institution*	Result
J.D. Taylor	BM(NH), U.K.	<i>Thais clavigera</i>
J. Sigurdsson	Zoology Dept., NUS	<i>T. luteostoma</i>
S.P. Kool	MCZ, Harvard Univ.	<i>T. luteostoma</i> (= <i>T. kieneri</i> )

\*BM(NH) = British Museum (Natural History);  
 NUS = National University of Singapore;  
 MCZ = Museum of Comparative Zoology.

### 2.3.2 Imposex Distribution and Measures

The body tissues of male and imposex female *Thais luteostoma* are shown in Figures 2.6a and b, while their morphometric data are presented in Appendix 7.

Appendix 8 provides a set of imposex measures, and values for various indices are mapped in Figure 2.7. The imposex frequency (RFI) at the six sampling sites shows that imposex occurred at all sites where females were found. It can be seen in Appendix 8 that the four harbours have higher RPS values, both Population and Incidence, than Poka Shore, but the VDS index does not show a corresponding pattern.

The three dimensional frequency bar diagrams demonstrate patterns of imposex distribution within and among sites. The frequency of female penis length is plotted against the males (Figure 2.8) for each site. It appears that at the three main harbours, i.e., Yos Soedarso, Galala Tuna and Gudang Arang, there were higher frequencies of females with large penes than elsewhere. Incrementally-ordered bar diagrams of Individual RPS values (Figure 2.9) show considerable differences among sites in female penis bulk (relative to the males). Some females have penes considerably larger than mean values for males.

Figure 2.6a **Male body tissue of *Thais luteostoma*** [A. o = operculum; ft = foot; me = mantle edge; k = kidney; te = testis; B. lt = left tentacle; rt = right tentacle; pf = penial flagellum; p = penis; vd = vas deferens]

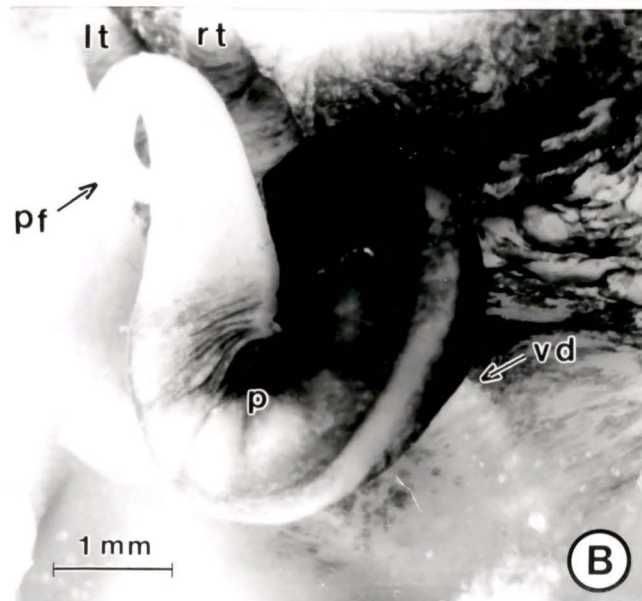
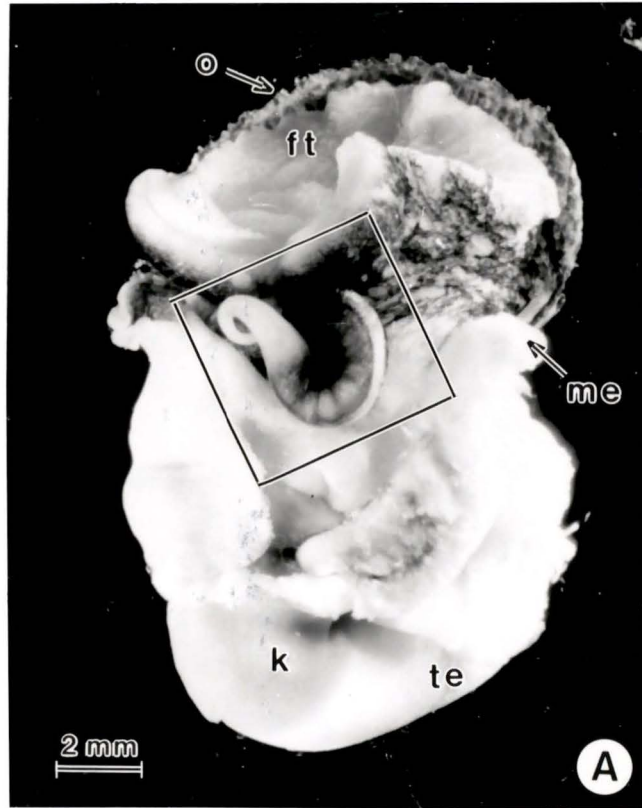


Figure 2.6b **Imposex female body tissue of *Thais luteostoma*** [A. o = operculum; ft = foot; pr = proboscis; t = tentacle; cg = capsule gland; dg = digestive gland; ov = ovary; sg = sperm-ingesting gland; B. p = penis; gp = genital papilla]

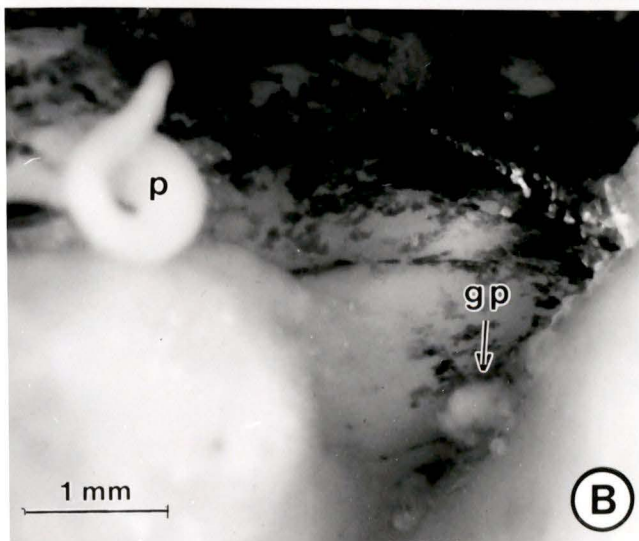
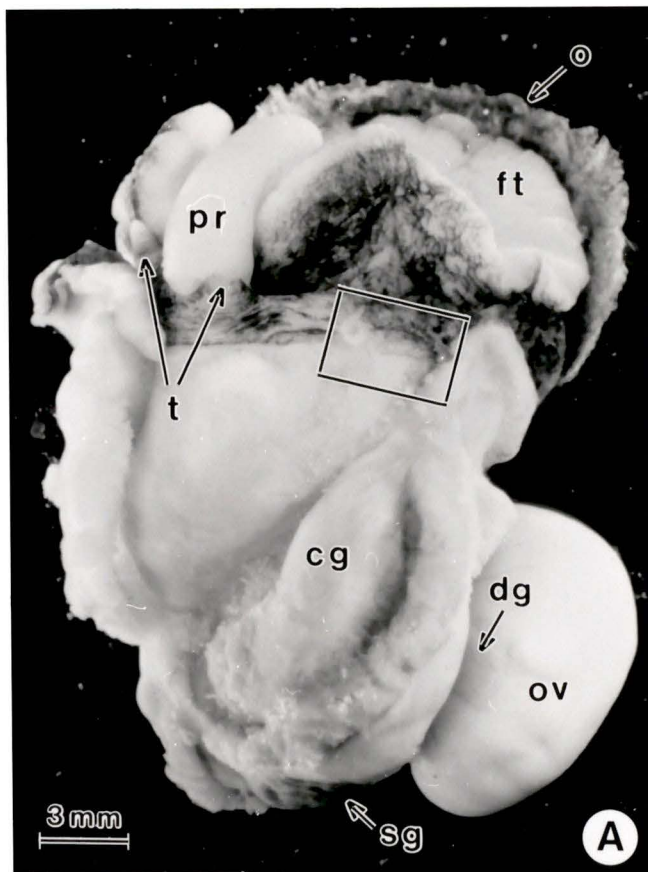


Figure 2.7 **Imposex occurrence and distribution in Ambon Bay, 1988** [GAH = Gudang Arang Harbour; YSH = Yos Soedarso Harbour; SRH = Slamet Riyadi Harbour; GTH = Galala Tuna Harbour; PFD = Poka Ferry Dock; PS = Poka Shore; the figures shown are the RFI/RPS<sub>pop</sub> values, with a note that no females were found at the Poka Ferry Dock]

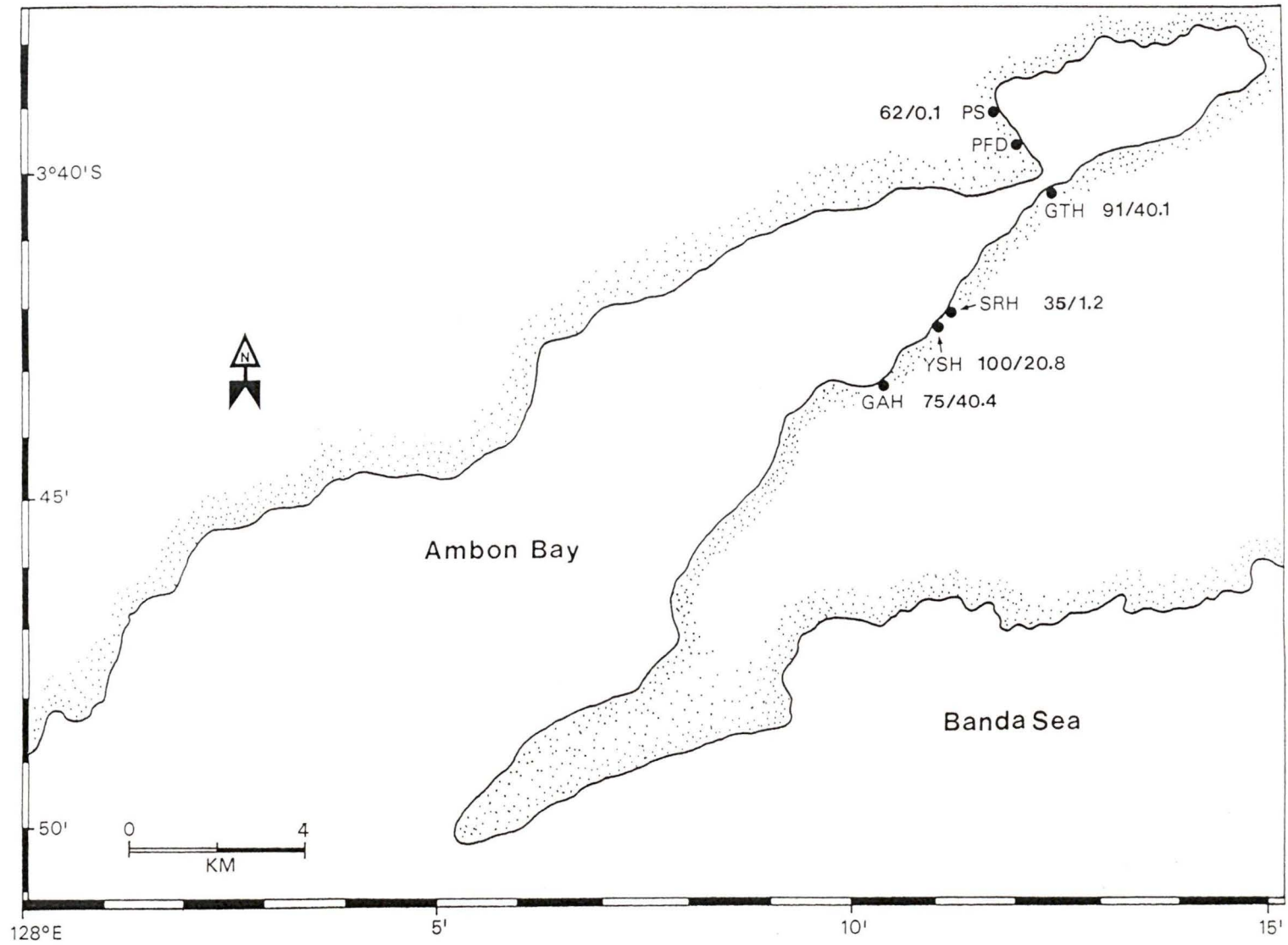
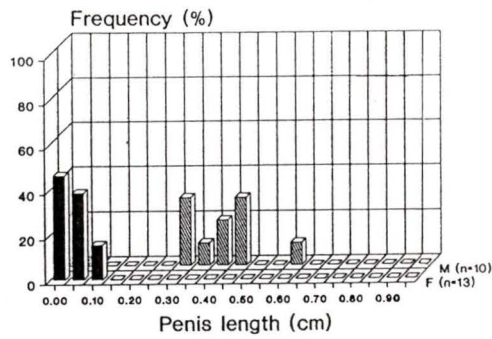
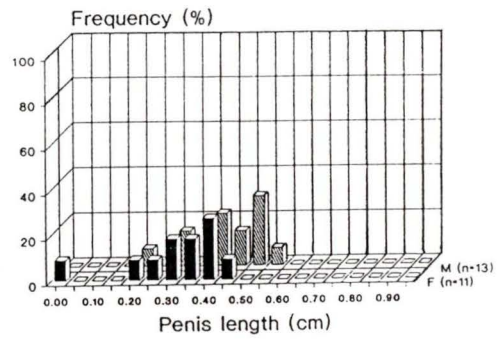


Figure 2.8 **Intrasite comparison of imposex occurrence in Ambon Bay, 1988**  
[M = *male*; F = *female*]

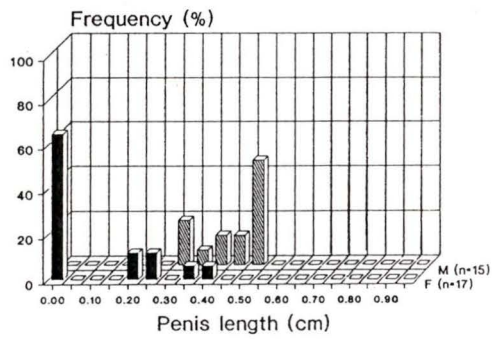
Poka Shore



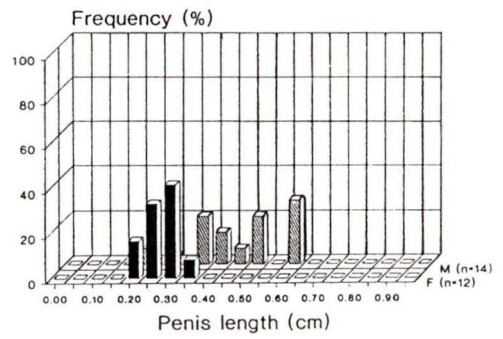
Galala Tuna Harbour



Slamet Riyadi Harbour



Yos Soedarso Harbour



Gudang Arang Harbour

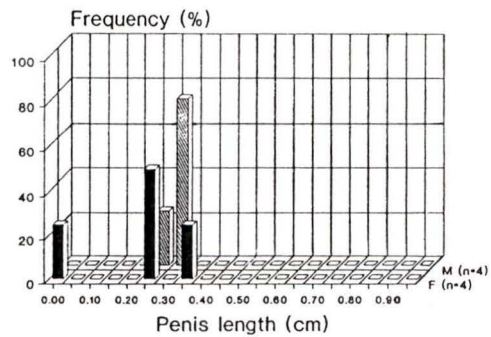
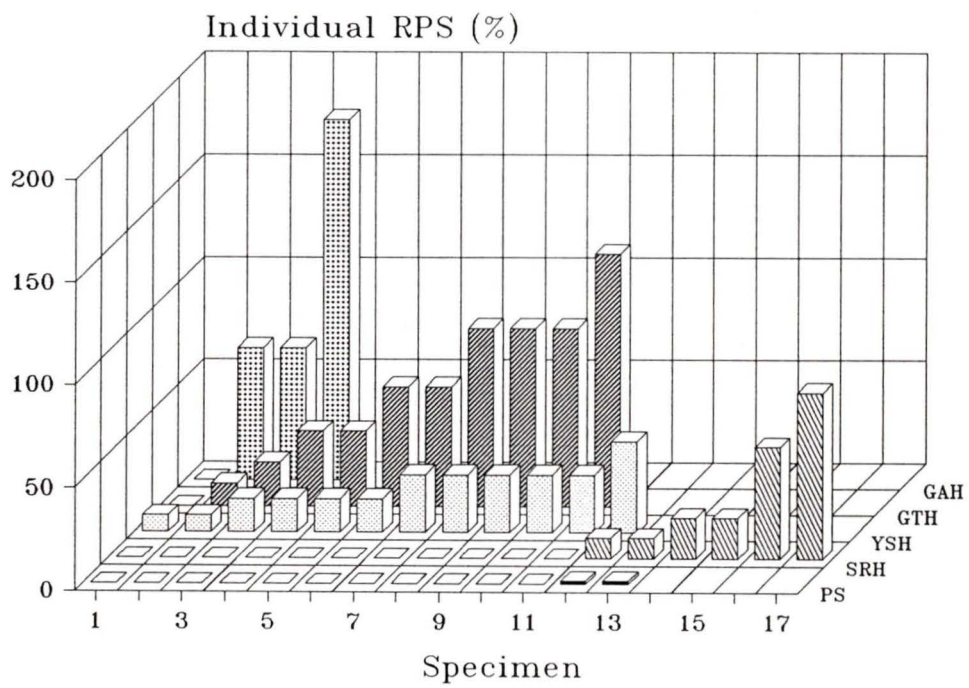


Figure 2.9 **Intersite comparison of imposex occurrence in Ambon Bay, 1988**  
[GAH = *Gudang Arang Harbour*; GTH = *Galala Tuna Harbour*;  
YSH = *Yos Soedarso Harbour*; SRH = *Slamet Riyadi Harbour*; PS =  
*Poka Shore*]



### 2.3.3 Normality and Homoscedasticity Tests

The summary statistics of female penis length data, and the results of normality and homoscedasticity tests are provided in Appendix 9. The Lilliefors Tests and the Probability Plots indicate that female penis lengths are normally distributed for Poka Shore, Galala Tuna Harbour and Gudang Arang Harbour, but there is a serious departure from normality for the Slamet Riyadi data set. The P-value for Yos Soedarso Harbour (0.052) is just above the significance level (0.05). Its probability plot, however, shows a normal distribution. The Bartlett Test shows high heteroscedasticity (heterogeneity of variance) among sites.

### 2.3.4 Tributyltin Use

Antifouling paints sold and used locally in Ambon City and its vicinity are shown in Appendix 10. Most of the antifouling paints sold in Ambon City and used in the Galala Slipway contained cuprous oxide as the primary agent. One paint, AF-Seaflo SP, which contains tributyltin as a primary agent, was only found at the slipway. This was an imported paint from Japan. It was rarely used due to its higher price. Accordingly, it appears that TBT antifouling paints are not used in Ambon City, except rarely at the Galala Slipway. A source of TBT contamination in Ambon Bay could be the Japanese fishing fleets, which regularly visit the Galala Tuna Harbour.

## 2.4

## Discussion and Conclusions

2.4.1 **Imposex and Tributyltin at Ambon**

This reconnaissance survey of neogastropod snails in a tropical bay (Ambon) reveals that at least one intertidal species, *Thais luteostoma*, shows imposex. In general, the morphological features of the body tissue of this species are similar to those of *Nucella lapillus* from the northern Atlantic (see Figure 2.1). Therefore, there is relatively little difficulty in gender determination, particularly with large specimens where the secondary sexual characters are often clearly seen. Accurate gender determination is an essential step in imposex investigations.

In imposex form, however, *Thais luteostoma* is very different from *Nucella* species. The presence of a long penial flagellum on males, which quite often coils, is common in this species. This poses a problem in standardizing penis length measures on live specimens. In practice, it is better to wait for a while after opening the mantle cavity until the penial muscle relaxes and then to start measuring. In placing the scale, contact with the penis should be avoided, particularly the flagellum, as it may contract.

None of the examined specimens showed the abnormalities in penis form recorded in *Nucella lapillus* around southwest England, such as bifurcation (Bryan *et al.*, 1986). The vas deferens in female *Thais luteostoma* was not a prominent feature as in *Nucella*. It was not an easy parameter to measure due to frequent difficulty in determining the presence and form of the vas deferens.

The RPS index of Bryan *et al.* (1986), which includes the non-imposex females, does not show how large is the approximate actual mean of female penes compared to that of males in the same population. This original RPS index (Bryan *et al.*, 1986) is termed here the Population RPS since it shows the average level of

imposex intensity for all the females in a population. The second RPS index calculated (the Incidence RPS) provides a measure from only females affected. Therefore, if all females were affected (i.e., 100% imposex incidence), the two RPS indices are identical. In populations with less than 100% incidence of imposex, the Incidence RPS provides a greater range of values hence is potentially a more sensitive measure.

The relationship between the Population RPS ( $RPS_{pop}$ ), the Incidence RPS ( $RPS_{inc}$ ) and the Relative Frequency of Imposex (RFI) is expressed by the following formula:

$$RFI = \left[ \frac{RPS_{pop}}{RPS_{inc}} \right]^{1/3}$$

(see Appendix 11 for its derivation). This empirical relationship illustrates how the two RPSs are related to the imposex frequency. Besides the VDS index, the Bryan index (i.e., Population RPS) and imposex frequency are the two other known imposex indices. If the incidence of imposex is less than 100%, by using this relationship (simplified as  $RPS_{inc} = RPS_{pop}/RFI^3$ ), the Incidence RPS can be calculated directly from the available values of the two indices, thus, providing additional information about the actual patterns of imposex intensities from the other neogastropod species or sites (local populations).

Since the Bryan index (Population RPS) is a ratio of the mean penis bulk of females to that of males, the intensity of imposex can also be expressed graphically so that the degree of overlapping between distributions of the penis length of females and males, and their patterns, can be clearly seen. Figure 2.8 shows the imposex occurrence within each site.

It appeared that Poka Shore had relatively low incidence and intensity of imposex compared to Slamet Riyadi, Gudang Arang, Yos Soedarso and Galala

Tuna Harbours. The shore is located in the inner part of the bay and is 'clean'. However, the coastal waters around it are not free from the traffic of deep-sea vessels, which are suspected as the sources of TBT contamination. Since TBT is more concentrated in the sea surface microlayer (Cleary and Stebbing, 1987), it can be washed ashore by waves and contaminate the shore life.

Slamet Riyadi Harbour showed low incidence of imposex, but at high actual intensity. This pattern is shown by its high Incidence RPS (26.2%) relative to its Population RPS (1.2%; see Appendix 8). This suggests that there may be differences in individual susceptibility. Bryan *et al.* (1988) found that in response to TBT, the penis growth increases more rapidly if a small penis is already present. Bryan *et al.* (1987) also showed that the juvenile stages of *Nucella lapillus* are more sensitive to TBT, a causative agent of imposex, than the adults. The affected females of *Thais luteostoma* at Slamet Riyadi Harbour ranged from 3.8 to 4.4 cm length and 2.2 to 2.6 cm width, while the ranges of the whole sample are 2.3 - 4.6 cm length and 1.7 - 3.3 cm width. Thus, larger snails show high intensity of imposex suggesting that this species may be long-lived, and the imposex was induced in the past. Another possible cause for this phenomenon is the uptake of TBT from the diet. It was shown that the diet may contribute almost 50% of the TBT body burdens in natural populations of *Nucella lapillus* subjected to life-long exposure (Bryan *et al.*, 1989b)

Gudang Arang Harbour has been formally closed for about 10-15 years. During this period, sometimes there were vessels anchored near the dock while waiting to enter the main harbour, Yos Soedarso. High imposex intensity found at this site seems to be caused by a contaminant derived from the anchored vessels. Adema *et al.* (1988) showed that a docked or anchored vessel is a source of continuous release of butyltin compounds from antifouling paints.

A third RPS index called Individual RPS has been calculated as the penis bulk (i.e., cubed length) of each individual female expressed as a percentage of the mean penis bulk of males. Figure 2.9 compares the occurrence of imposex (both imposex incidence and intensity) among sites based on this index. Figures 2.8 and 2.9 and the other measures of imposex (see Appendix 8), reveal that the occurrence of imposex is highly localized to Galala Tuna, Yos Soedarso and Gudang Arang Harbours (see Figure 2.7). Sites with low degrees of imposex, such as Slamet Riyadi Harbour and Poka Shore are nearby, suggesting that imposex in *Thais luteostoma* shows a 'hotspot' pattern as it does elsewhere.

Lack of randomization in the field collections, primarily at the harbours, could possibly have biased the results in some way. It should be noted, however, that specimens were collected as they were seen by the divers, and imposex is not visible in the field.

The Bartlett Test is sensitive to non-normality (Sokal and Rohlf, 1981). Its result shows high heteroscedasticity of the female penis length among sites. In the summary statistics of the data, it can be seen that the variances of Poka Shore and Yos Soedarso Harbour are quite different from those of Galala Tuna Harbour and Gudang Arang Harbour (see Appendix 9). Therefore, even if the non-normal data set (Slamet Riyadi Harbour) were excluded from the calculation, the result of the Bartlett Test would remain the same. Accordingly, no statistical test for imposex intensity comparisons among sites was made. The high percentage of cells (>20%) with low expected frequency (<5.0) also precluded  $\chi^2$  testing for independence of imposex incidence on site. Nevertheless, the Incrementally-ordered Individual RPS diagram, specially developed to overcome these problems, effectively demonstrates widespread imposex occurrence in Ambon Bay. The pattern of imposex distribution, as shown by the diagram, suggests that the imposex occurrence in the bay associates with marine traffic.

The whelk imposex in Ambon Bay occurred most frequently and in most intense form (by all indices) at two small harbours serving foreign and domestic vessels, i.e., Galala Tuna and Yos Soedarso Harbours. Thus, there is a reason to suspect that the imposex is derived, as in other regions, from boat and ship TBT-antifouling paints.

The surveys of marine paint availability in Ambon show that there are three active agents, i.e., cuprous oxide, triphenyltin and tributyltin (TBT), in antifouling paints used locally (see Appendix 10). TBT is little used due to its higher cost relative to copper-based paints. Copper-based paints do not induce imposex (Smith, 1981a; Bryan *et al.*, 1987) nor do triphenyltin compounds (Bryan *et al.*, 1988). The visiting Japanese fishing fleets are strongly suspected to use TBT-based antifoulants routinely. It should be noted that tripropyltin, another known organotin compound that has some capacity to induce imposex (see Appendix 4), was not found during the surveys of marine paint use in Ambon.

The pattern of imposex distribution in Ambon Harbour in 1988 suggests that it is TBT-based around harbours, probably from foreign ships. This conclusion met the objectives of the 1988 reconnaissance, and generated a working hypothesis to be tested in the 1989 field season.

#### 2.4.2 Problems With Reconnaissance Surveys in New Areas

A number of biological and logistical difficulties appeared during the reconnaissance survey. The main problem was biological and comprised locating affected species easily, in large numbers and preferably intertidally for ease of collecting. Some species were available from hard surfaces of wharfs and docks (i.e., *Thais luteostoma*, *Mancinella* sp. and *Chicoreus* sp.), mangrove trees (i.e., *Morula* sp.) and low intertidal sediments (i.e., *Nassarius* sp.). Collecting at harbours

was found easiest by snorkeling divers at slack water, rather than by low tide collecting, due to the difficulties of access from the shore. However, it is not easy to randomize samples in such underwater collections.

A further biological problem was identifying the affected species. Specimens of this most widely distributed species were identified as *Thais kieneri* (Deshayes) based on limited taxonomic literature available at the field site and a time constraint. The specimens were then provided to Dr. John Taylor, British Museum (Natural History), who made the identification as *T. clavigera* (Kuster). Dr. Jan Sigurdsson, National University of Singapore, Department of Zoology, identified them later as *T. luteostoma* (Holten). Eventually Dr. Silvard Kool of the Harvard University, Museum of Comparative Zoology, identified the specimens as *T. luteostoma*, and noted that *T. kieneri* was a junior synonym.

Other species present were only identified to generic level. They are generally smaller and less accessible for collection than *Thais luteostoma*. Accordingly *T. luteostoma*, as a representative mid-tide relatively large whelk was the species of choice for further research.

## Chapter III

### FOLLOW-UP FIELD RESEARCH 1989 AT AMBON BAY

#### 3.1

#### Introduction

A second field sampling program was designed and implemented in 1989 following the reconnaissance survey of 1988. There were two objectives.

The first objective was to obtain further measures of imposex occurrence from some new sites to allow testing the hypothesis that imposex occurrence in Ambon Bay is likely to be derived from TBT antifouling paints, probably from the visiting Japanese fishing fleets. If this were true, then yet another marine traffic-associated pattern of imposex occurrence would be indicated. Locations chosen included a site with known high occurrence of imposex (Galala Tuna Harbour), a marine traffic site previously sampled from which no females were obtained (Poka Ferry Dock), and two new sites expected to have moderate and no occurrence, respectively. These were Galala Ferry Dock and Amahusu Shore (see Figure 3.1). Thus, the objective was to provide additional and improved data for interpretation.

The second objective was to obtain specimens for microscopy at the University of Victoria in order to refine the gender determining protocol under development (Ellis and Pattisina, 1990). Results are presented and discussed in Chapter 4.

## 3.2

## Materials and Methods

### 3.2.1 Site Selection and Field Collection

Field work was conducted from May 19 to 26, with sampling as shown in Appendix 12 and sites in Figure 3.1.

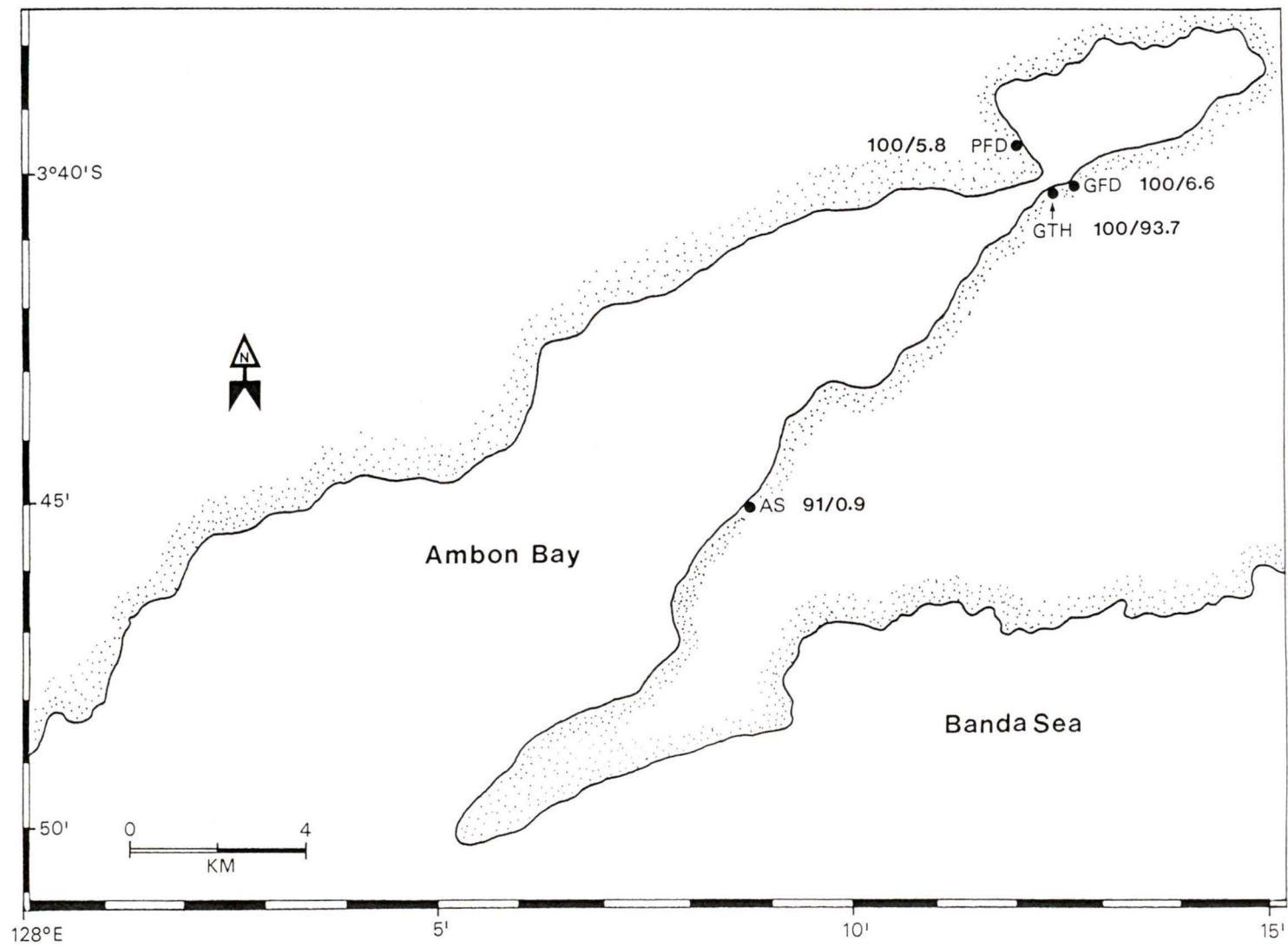
Galala Tuna Harbour was selected as the Test Site due to its known high occurrence of imposex in 1988. This harbour is periodically visited by Japanese fishing fleets. It also has a slipway where TBT is infrequently used.

Two sites were selected for high probability of moderate occurrence of imposex. Galala Ferry, a new site in 1989, is a ferry dock about 0.5 km to the northeast of Galala Tuna Harbour. Poka Ferry Dock is across the bay from Galala Ferry.

The second new site in 1989, Amahusu Shore, was selected as a Reference Site, with expected low occurrence of imposex, due to its remoteness from harbour facilities. Amahusu is located on the southeastern coast of the outer bay, about 4 km to the southwest of the Ambon Harbour area (see Figure 3.1).

Specimens were selected in the field as being the same species surveyed in 1988, i.e., *Thais luteostoma*. At Galala Tuna Harbour, all visible specimens on wooden pilings under the dock were collected in the daytime by a snorkel diver, as in 1988. At the other sites, specimens were collected by hand at low tide. Randomization was not possible in the underwater collections, and all specimens seen were collected. It was also not necessary at some sites due to the low abundance of specimens (about 30). All specimens seen were collected.

Figure 3.1 **Imposex occurrence and distribution in Ambon Bay, 1989** [AS = *Amahusu Shore*; GTH = *Galala Tuna Harbour*; GFD = *Galala Ferry Dock*; PFD = *Poka Ferry Dock*; the figures shown are the *RFI/RPS<sub>pop</sub>* values]



Specimens were kept in plastic bags or buckets filled with seawater, and returned to the laboratory for preliminary examination. Body tissue examination of imposex was done on a subsample of 10 specimens from each site due to time constraints. The body tissues were then kept in Bouin fixative (Humason, 1979) for later gender confirmation by sectioning and microscopy. The rest of the specimens were preserved in 4% formaldehyde solution (see Appendix 12) and brought back to the University of Victoria for later examination and gender confirmation (discussed in Chapter IV).

### 3.2.2 **Imposex Measures and Data Analyses**

The procedures of the 1988 reconnaissance survey were followed, with some minor changes, i.e., shell height and the Vas Deferens Sequence (VDS) were not recorded. Shells were generally overgrown, so that height measures were inaccurate, and the VDS had been a very time-consuming and unreliable measure in 1988. Measures, therefore, consisted of presence-absence of imposex (RFI) and penis length measures. These were determined as described in Chapter 2 and provided the basis for calculating the three indices of Relative Penis Size (Population RPS, Incidence RPS and Individual RPS).

Data from the specimens examined alive in the field (female  $n = 1$  and  $2$ , see Appendix 13) were not included in imposex comparison. Imposex calculations were made as in 1988, and the data are presented by similar graphics; three-dimensional frequency bar diagrams of female-male penis length distributions and the incrementally-ordered Individual RPS. Normality and homoscedasticity tests were only executed for the data from the preserved specimens.

### 3.3

## Results

### 3.3.1 **Imposex occurrence and distribution**

Data for shell length and width, and penis length are provided in Appendix 13. The imposex measures in Ambon Bay for both live and preserved specimens are shown in Appendix 14. The two types of specimens show corresponding imposex measures. However, the live specimen data are not intended for intersite comparisons of imposex. The distribution of imposex occurrence, based on the preserved specimen data and expressed by the RFI and RPSpop indices, is mapped in Figure 3.1.

At all Test Sites, all females showed imposex. The intended Reference Site, Amahusu Shore, was not free of the abnormality but showed 91% incidence of imposex. Note that at RFI = 100%, the Population and Incidence RPS values are identical (see Appendix 14).

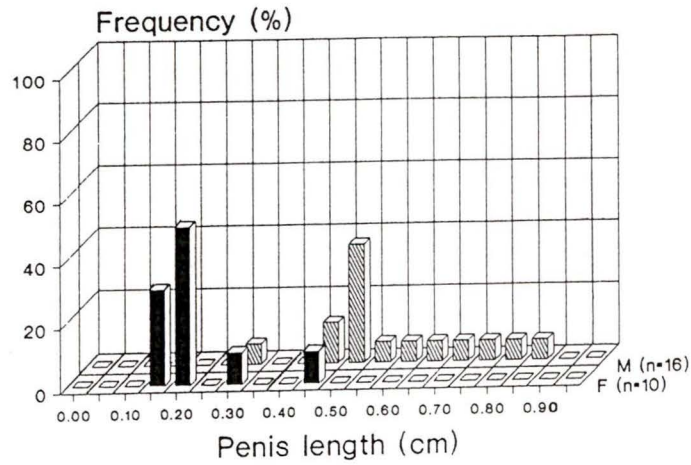
Penis length frequencies of females plotted with those of males (Figure 3.2) and the incrementally-ordered plots of Individual RPS (Figure 3.3) showed differences among sites. Galala Tuna Harbour had high imposex levels (Appendix 14 shows RPS value of 94% for preserved specimens) compared to the other three sites (RPS values ranging from 0.9 to 6.6%). Galala Ferry and Poka Ferry Docks had RPS values of 6.6 and 5.8%, respectively. Amahusu Shore, the chosen Reference Site, had the lowest RPS value of 0.9%.

### 3.3.2 **Normality and Homoscedasticity Tests**

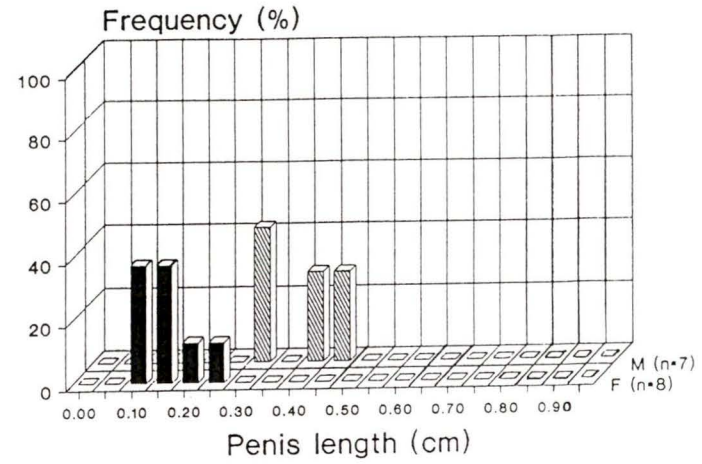
Appendix 15 showed the results of normality and homoscedasticity tests. The Lilliefors Test and Probability Plots indicated that the Poka Ferry data were non-normal, while the Amahusu Shore data showed a serious departure

Figure 3.2 **Intrasite comparison of imposex occurrence in Ambon Bay, 1989**  
[M = *male*; F = *female*]

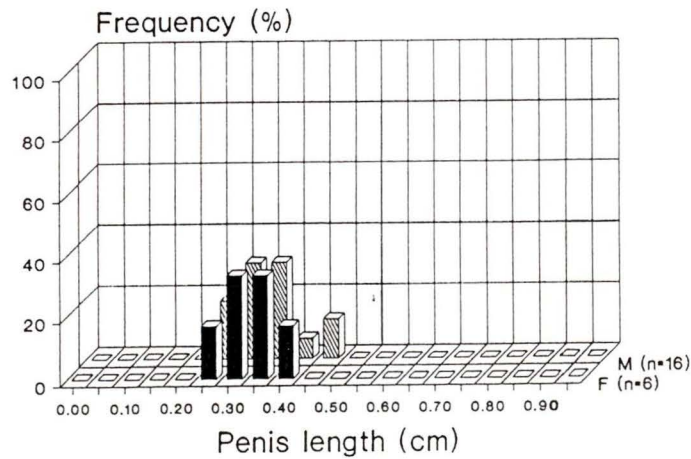
### Poka Ferry Dock



### Galala Ferry Dock



### Galala Tuna Harbour



### Amahusu Shore

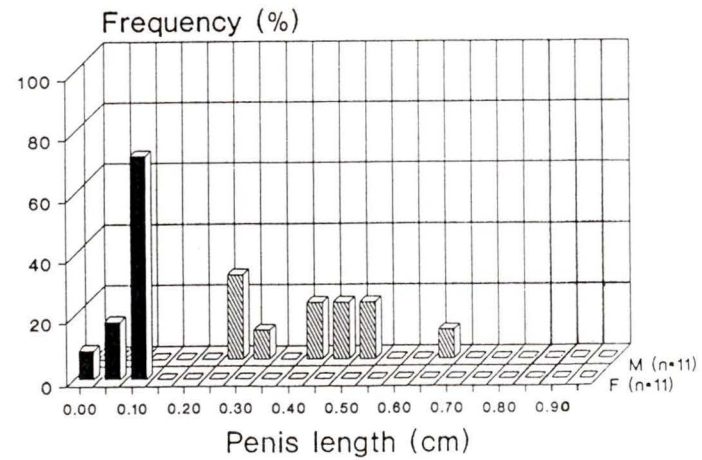
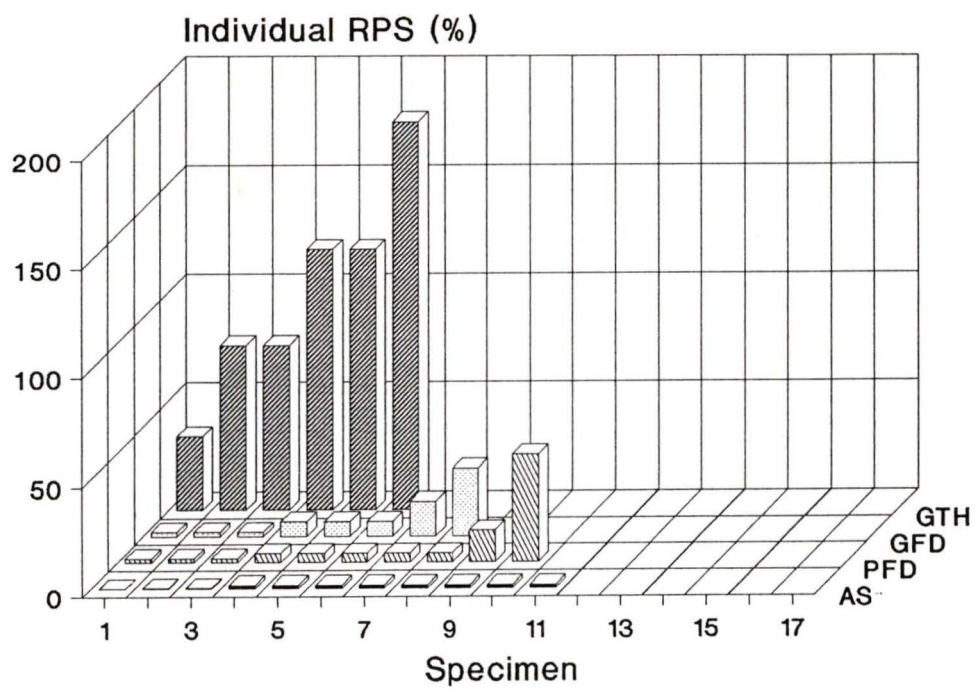


Figure 3.3 **Intersite comparison of imposex occurrence in Ambon Bay, 1989**  
[GTH = *Galala Tuna Harbour*; GFD = *Galala Ferry Dock*; PFD = *Poka Ferry Dock*; AS = *Amahusu Shore*]



from normality (see the Probability Plot in Appendix 15). The result of the Bartlett Test showed heteroscedasticity for the four sites.

### 3.4

### Discussion

The 1989 data confirmed the presence of neogastropod imposex in Ambon Bay, and provided measures from additional sites, including one chosen for its remoteness from harbour traffic.

The high incidence of imposex at all sites (91-100%) extends the results of the 1988 survey to show that the phenomenon is widespread with 'hot spots' at high traffic areas. This agrees with studies elsewhere (see Section 1.1.3).

The Population and Incidence RPS indices between them show recurring patterns of highest values at Galala Tuna Harbour, and least values at the remote Amahusu Shore. Galala Tuna Harbour provides docking space for deep-sea vessels, including visiting foreign ships.

The normality and homoscedasticity tests were executed on the 1989 data for a comparison with the result of the 1988 data. No further statistical tests were attempted. Instead, the Individual RPS bar diagrams was used for intersite comparison of imposex as it has effectively shown the difference in imposex occurrence among sites for the 1988 data.

The graphical display of the Individual RPS diagrams shows a gradient of increasing imposex intensity with increased marine traffic. The three sites away from the hotspot, Galala Tuna Harbour, had lower intensities. This suggests that the widespread imposex occurrence in Ambon Bay is not random, but is associated with marine traffic. The same conclusion was achieved in the 1988 survey. This

supports the 1988 hypothesis that imposex occurrence in Ambon Bay, as elsewhere (see Section 1.1.3), is derived from ship TBT-antifouling paints. However, this conclusion needs testing through chemical analyses.

## Chapter IV

### GENDER DETERMINATION

#### 4.1 Introduction

The initial examination of specimens in Ambon in 1988 and 1989, and elsewhere in southeast Asia (Ellis and Pattisina, 1990) showed that there was considerable difficulty in gender determination for some neogastropod species. Accordingly, the 1989 specimens were brought to the University of Victoria for appropriate gonad examination by microscopy.

There were two objectives of this histological gonad examination. The main objective was to reexamine specimens which could not be sexed by secondary sexual characters. The second objective was to confirm that the presence in squashes of microscopic translucent oval-shaped structures (designated here as "ovoids") indicates the female sex. If this were confirmed then gender determination by body tissue examination (for secondary sexual characters) plus gonad squashes would provide a good protocol, so that time-consuming gonad sectioning would not be necessary.

#### 4.2 Materials and Methods

##### 4.2.1 Specimens

Specimens for gender confirmation consist of 1988 and 1989 specimens preserved in buffered formalin, 4% formaldehyde and Bouin fixative. All gonads

from the previous sexually undeterminable specimens were examined, except for the broken or small gonads.

#### 4.2.2 Gender Confirmation

Two methods of gonad examination were used: microscope squashes and histological sections.

Gonad squashes under light microscopy provided presence-absence records of translucent oval-shaped structures ("ovoids"). These are provisionally concluded to be ovarian storage granules (Reid, *personal communication*, 1991). They are only found in gonads containing oocytes. Three samples were taken from the gonad, or 5-6 samples from its outer edge if there was no clear separation between gonad and digestive gland.

Gonads of 58 preserved specimens were first examined by squashing. The gonads containing ovoids were recorded as females, and those lacking ovoids were recorded as males. Subsequently, all the gonads were embedded in paraffin, and cut into 10  $\mu\text{m}$  thick cross-sections, stained with Harris hematoxylin and counterstained with Putt's eosin (Putt, 1972).

### 4.3

### Results

Results of the gonad squash and gonad histological section procedures are shown in Appendix 16 and Figure 4.1.

Figure 4.1 shows presence of the translucent ovoids (A) in the squash preparations, and in dark form (red-stained, C) in histological sections. Ovoids were present in virtually all squashes (91%) examined from specimens previously designated as females based on the secondary sexual characters. In the example

male preparations, in all cases there were no ovoids present, but spermatozoa (purple-stained, D) could be seen in some sections (60%). In both sexes, the digestive gland tissues were pink-stained. Almost all the sexually undeterminable specimens (87.5%) based on the body tissue examination for secondary sexual characters remained undetermined.

#### 4.4

#### Discussion

The gender determinations made on live specimens were supported by the gonad squash microscopy and histological sectioning. The presence of translucent ovoids matched the occurrence of female secondary sexual characters, with the exception of two specimens not identifiable to gender. There were no instances of live specimens identified as males, with subsequent determination of the ovoids.

The ovoids had previously been noted in females by Ellis and Pattisina (1990), and were also shown in a section of ovary by Gibbs *et al.* (1988). Their designation as ovarian storage granules requires histochemical confirmation.

Based on the results of this gonad examination under microscopy, a protocol for gender determination in gonochoristic neogastropods was derived as seen in Figure 4.2. In the majority of large specimens, the secondary sexual characters, in particular the sperm-ingesting gland, are reliable for gender determination (see also Section 5.2). In this research, the sex of almost all specimens determined by the body tissue examination were confirmed, while most of the sexually undeterminable ones remained unconfirmed.

Figure 4.1 **Gonad microscopy for *Thais luteostoma*: comparison between squash and section results** [A & B are the squash results, C & D are the section results; A & C = ovary (the "ovoids" are the oval-shaped structures), 485X and 195X respectively; B & D = testis (spermatozoa are only shown in the section result: D), 195X]

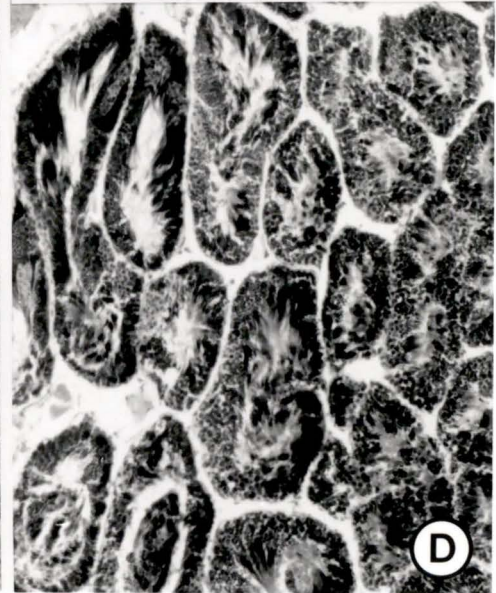
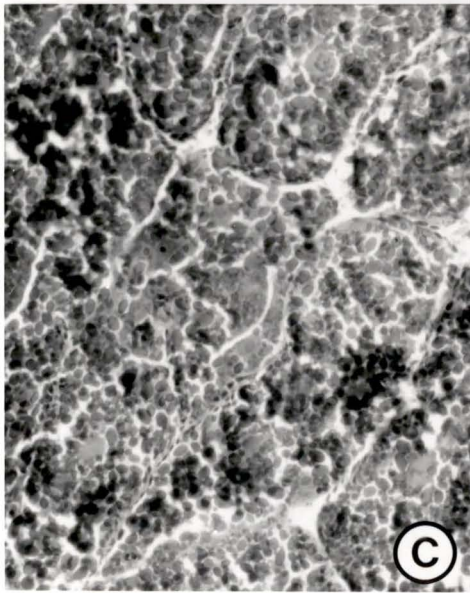
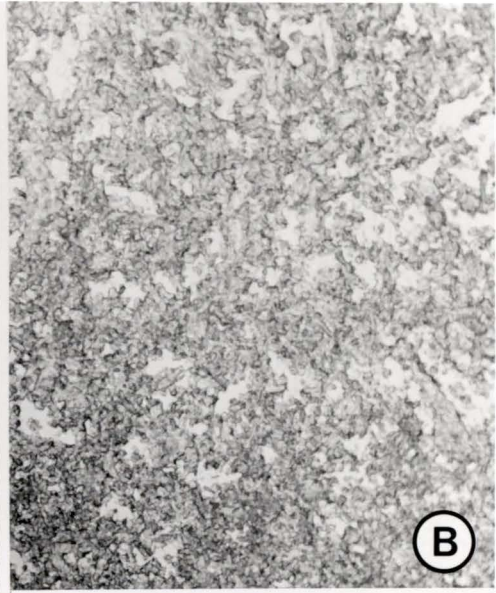
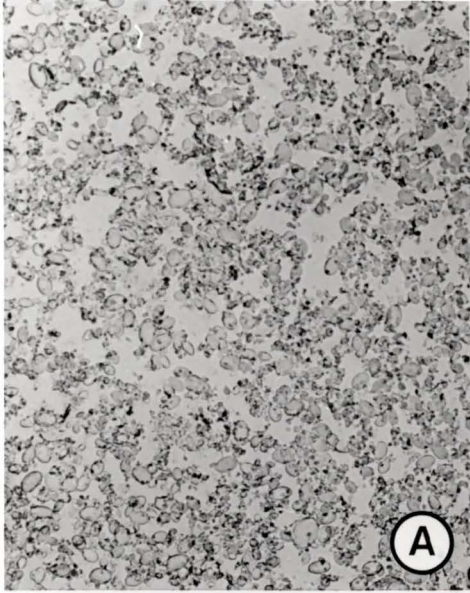
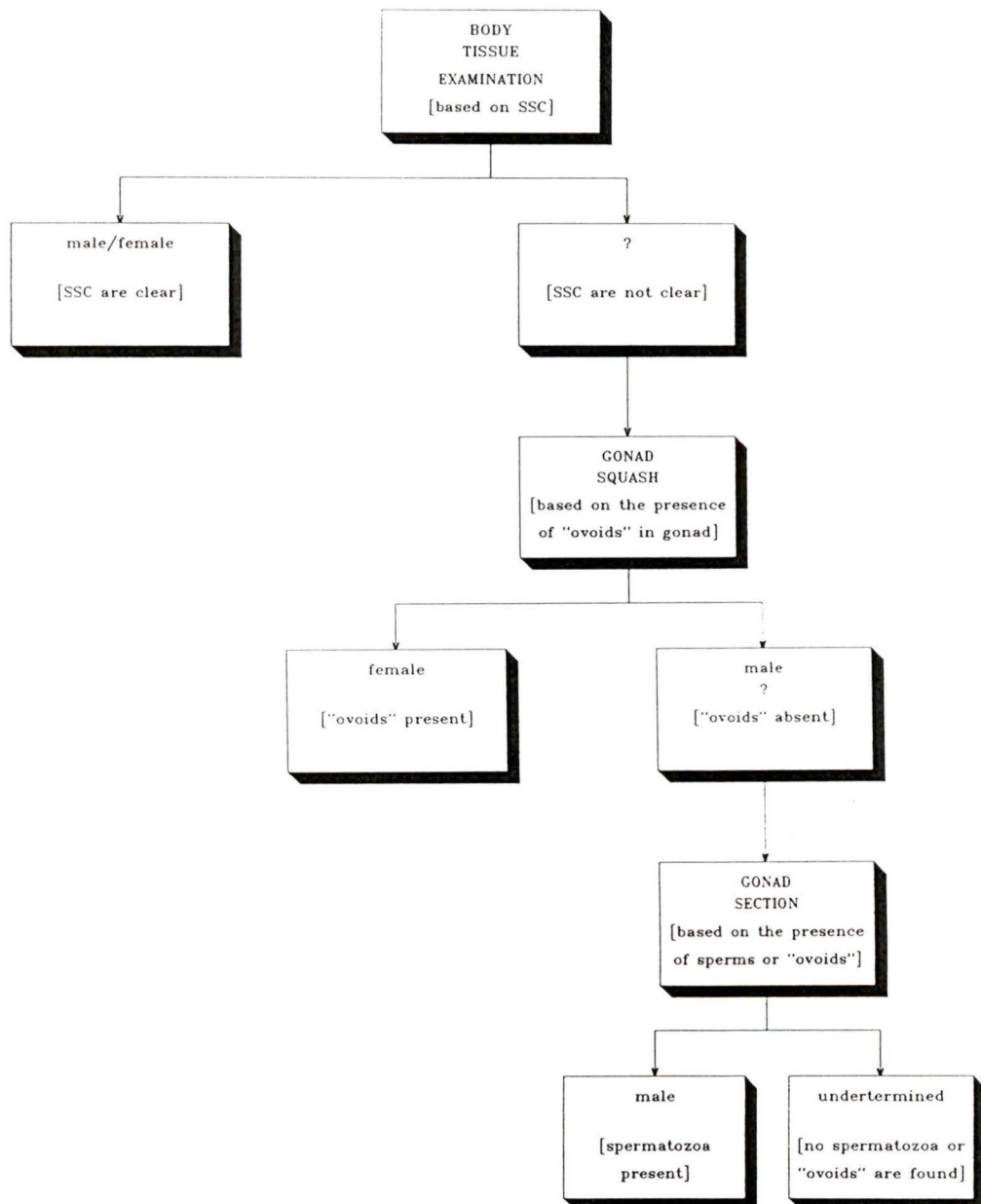


Figure 4.2 **Flow diagram for gender determination protocol** [SSC = *secondary sexual characters*]



## Chapter V

### GENERAL CONCLUSIONS AND RECOMMENDATIONS

#### 5.1 Imposex Data Analyses

The RPS index was presented by Bryan *et al.*(1986) as  $(\text{mean female penis length})^3 / (\text{mean male penis length})^3 \times 100\%$ . In algebraic terms, this simple form is identical to:  $(\text{mean female penis length}/\text{mean male penis length})^3 \times 100\%$ . The second form, however, is a shortcut calculation, and hence is more convenient.

Three of the indices used here provide measures of progressive sensitivity to imposex. The frequency measure (RFI) appears to be most sensitive, as generally values from 0% to 100% can occur with low values for penis length. In other words, imposex incidence may be high, but at low intensity levels.

The RPS index is a mean ratio between female (numerator) and male (denominator) penis lengths. The frequency of abnormal female penis (i.e., RFI) appears to depend on the degree of TBT contamination. At lower frequency, normal females (i.e., females with zero penis lengths) are to be excluded from the calculation of the  $RPS_{inc}$ . As a consequence, the numerator mean of the RPS increases. The result is that the value of the  $RPS_{inc}$  is always higher than that of the  $RPS_{pop}$ , if the  $RFI < 100\%$ . The difference between the two RPS indices of a population becomes smaller as the RFI approaches 100% (i.e., increasing number of the affected females) and is zero (i.e.,  $RPS_{inc} = RPS_{pop}$ ) when the  $RFI = 100\%$  (i.e., all the females have been affected). In an ecological sense, a substantially higher  $RPS_{inc}$  than the  $RPS_{pop}$  (i.e., high  $RPS_{inc}$ , but low  $RPS_{pop}$ ) could indicate that the local population is in a recovery process from TBT contamination. Some

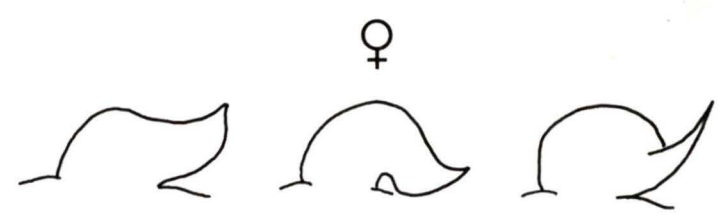
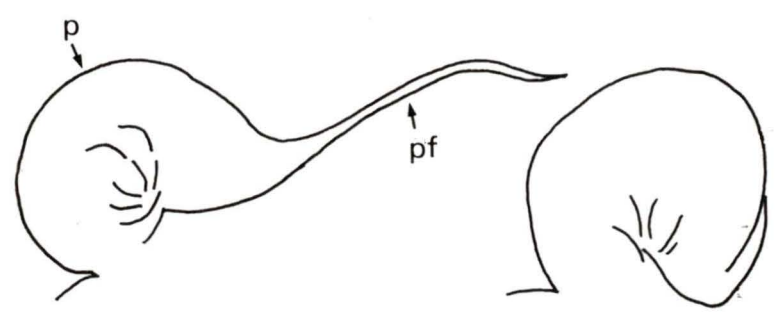
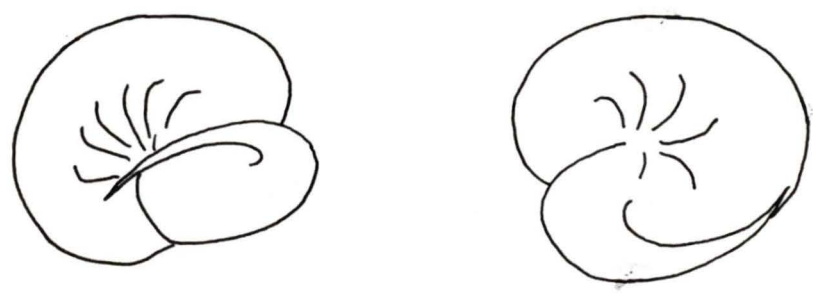
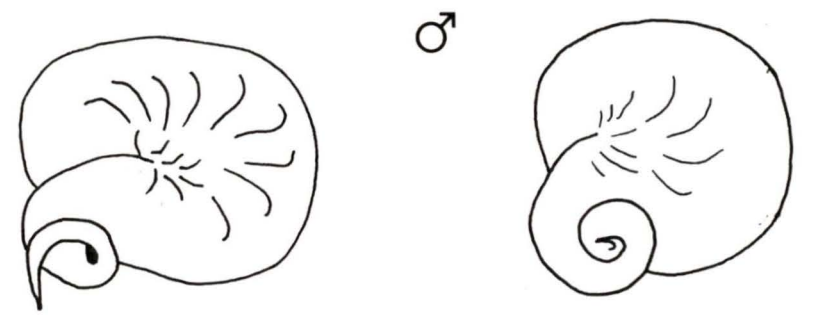
ecological interpretation of this "threshold" phenomenon as observed at the Slamet Riyadi Harbour has been discussed in Section 2.4.1. Long-term monitoring is needed to study the time pattern of imposex occurrence at this site.

Penis size does not indicate a female's reproductive competency, but it is a simple parameter to measure (Smith, 1981a; Gibbs *et al.*, 1987; Short *et al.*, 1989). In *Thais luteostoma*, penis size is more reliable compared to the vas deferens sequence. The expression of imposex occurrence in Ambon Bay in both years' surveys was mainly based on the RPS index. This index was found practical for monitoring TBT contamination (Short *et al.*, 1989). However, since the index is a mean ratio, it is subject to extreme penis length depending on the penis morphology that could vary according to species. Even in one species, like *T. luteostoma*, various degrees of contraction of a penis with a long flagellum could increase variability in penis length measures. Figure 5.1 shows various forms of male and female penes of *T. luteostoma*.

Gibbs *et al.* (1987) found that in severely contaminated areas, the male penis of *Nucella lapillus* was often grossly deformed. This deformation includes enlargement and bifurcation which usually increase the male penis length, resulting in underestimation of the imposex intensity (Bryan *et al.*, 1986).

A contradictory case is found in *Thais luteostoma*. Many of the male penes of this species have a long slender flagellum at the tip. The flagellum is usually 2-5 mm long, but it could be as long as 8 mm, about twice the average length of the large main penial body (see Figure 5.2). The flagellum is very contractile. In almost all live specimens examined it was coiled. This can increase the RPS values, and the imposex intensity is thereby overestimated. To reduce this effect, penis lengths were measured without contacting the relaxed penial muscle and all the measures were taken as homogeneously as possible, i.e., the diameter of the relaxed coiled penis.

Figure 5.1 **Various forms of male and female penes of *Thais luteostoma***  
[p = *penis* (main penial body); pf = *penial flagellum*]



This was the diameter of a recurved main penial body, i.e., the part of a penis excluding the flagellum (see Figure 5.1). The problem, however, is the obscure transition between the anterior end of the main penial body and the flagellum, as observed in most male specimens. However, since the coiled male penis form is common in this species its effect on imposex interpretation, at least for intersite comparison, could be ignored. Extreme penis lengths were recorded. One extreme value of 1.5 cm was excluded from the calculations. The female penis of *T. luteostoma* could also be found in a coiled form, but it was rare.

The VDS index was found useful in assessing the impact of TBT on the population ecology of affected species (Gibbs and Bryan, 1986; Gibbs *et al.*, 1990; Bright and Ellis, 1990; Stewart *et al.*, *in press*). In *Thais luteostoma*, however, the vas deferens is not a prominent feature. In such a case, determination of the VDS stages is not only very difficult, but may be affected by light reflection from very tiny muscular ridges. Unless high resolution microscopy is employed, the VDS index protocol is impractical in *T. luteostoma*.

Non-normality and heteroscedasticity are common in biological data (Day and Quinn, 1989). The normal distribution can normally be approached by increasing sample size; how large an appropriate sample size should be depends on the nature of the measured variable (Sokal and Rohlf, 1981). In a penial-based imposex measurement on live specimens, contraction of penial muscle is a source of error, not only in the quantification of the degree of imposex intensity, but also in the sample examination for normality. The contraction of this very contractile organ changes its form, resulting in variable penis lengths. Therefore, a large sample size seems necessary for a rigorous imposex data analysis. The appropriate sample size could vary according to species due to the differences in penis morphology. It should be noted that the sex ratio is important to consider in order to get a balanced number of each sex.

The basic problem in field collection on Ambon Bay shores is that it is often difficult to get a large number of neogastropod specimens. This is due to the isolated habitat pattern where a local population (enclave) consists of a few specimens only. Most sites sampled in this research provide too few specimens to support a rigorous statistical analysis. In the 1988 survey, for example, after about an hour's exploration at Gudang Arang Harbour, the diver only collected 8 specimens. In the 1989 survey, after all the rocky habitat beside the Galala Ferry Dock was intensively searched, only 26 specimens were collected. The developed Individual RPS bar diagrams offers a useful technique for analyzing small-sample imposex data.

The incrementally-ordered Individual RPS diagrams provide an alternative solution for intersite comparison of imposex occurrence. This Individual RPS-based graphical method also compare both imposex incidence and intensity simultaneously. This graphical method should be especially useful in imposex surveys where local populations of whelks are small, such as on many tropical shores and in southwest England where the affected populations of the Dog-whelk, *Nucella lapillus*, are declining.

Thus, for small size populations (enclaves) of neogastropods, the most appropriate protocol for quantification of imposex determinations is to obtain sets of presence-absence records for RFI calculations, penis length measures for RPS calculations and graphical displays such as the Individual RPS bar diagrams.

## 5.2

### **Specimen Examination and Gender Determination**

Accurate gender determination is essential for accurate surveys of imposex. Presence of a penis cannot be used for determining males. It must be established that the female penis is sufficiently distinctive in form from the male that the two

forms are diagnostic for male and female respectively. If this is not so, then other characters must be used for determining the sex of specimens.

Presence of a sperm-ingesting gland (see Figure 2.6b) is the most reliable female character for *Thais luteostoma*, as in *Nucella lapillus* (Gibbs *et al.*, 1987, 1988). The genital papilla is rarely observed, while the bright yellow colours of the capsule gland and ovary are not consistent. In many specimens, but not all, the male penis has a long (2-5 mm) flagellum as its tip. The direction at which the vas deferens (if it is present) emerges from the penial base is a good means for distinguishing a male from an imposex female. The male vas deferens usually descends to the right side and turns posteriorly, while the vas deferens of an imposex female is directed to the right towards the genital papilla. Moreover, the vas deferens of the male is usually prominent, while that of the female is not easily observed and traced.

In general, large specimens can be sexed easier than small specimens (see Appendices 12 and 13). This is probably due to sexual immaturity of the smaller size specimens.

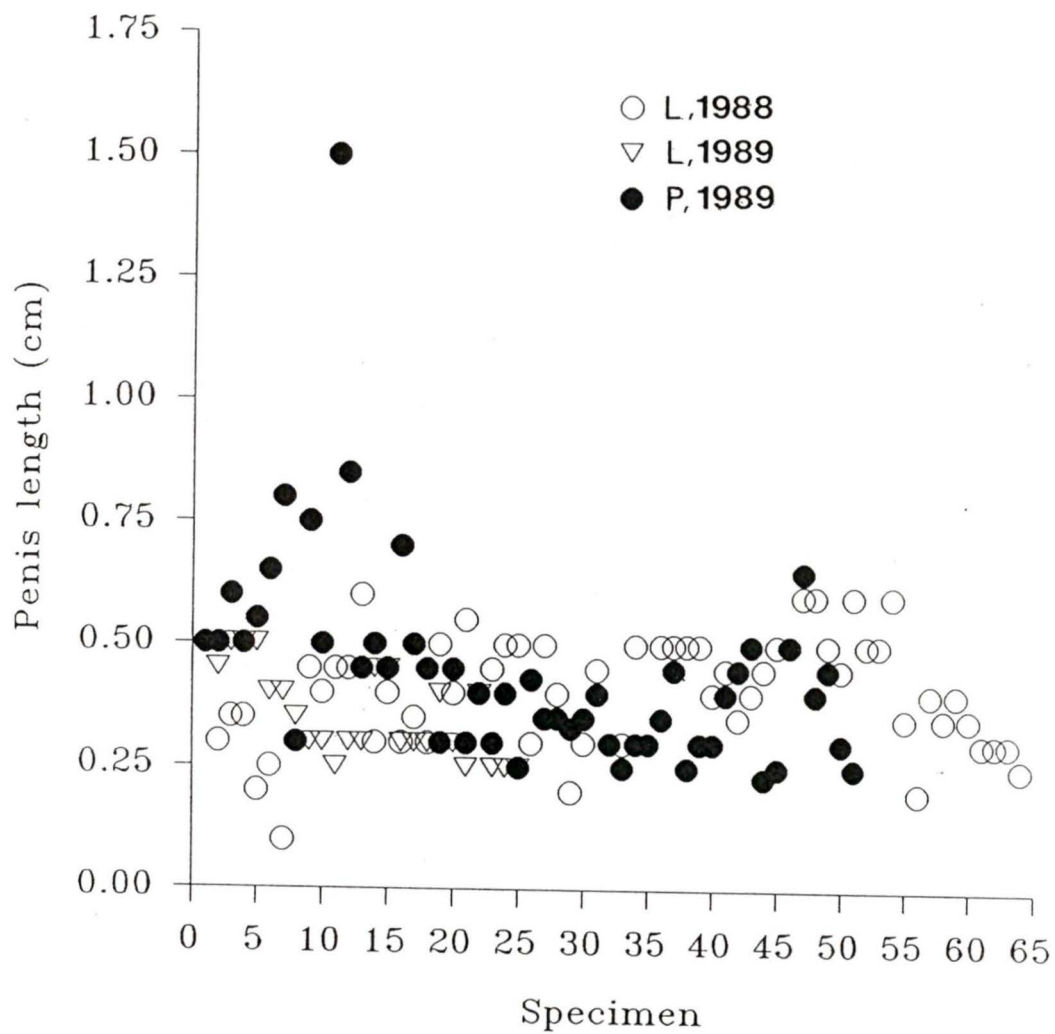
With those specimens collected and preserved in their shells (without cracking) in 4% formalin for later examination, it was found that the soft tissues of large specimens had generally not preserved properly. As a consequence, the body tissues were spoiled (the contents of the capsule glands and the junction part between the body and gonad were often found watery) and easily broken apart when the shells were removed. In general, the capsule glands were faded. Some sperm-ingesting glands showed the same problem. Gender confirmation is normally desirable, and specimens for later laboratory examination should be removed from shells before preserving. Nevertheless in regions little known taxonomically, some specimens must be preserved with shells intact for later taxonomic study.

If few specimens are available, there can be a conflict of needs in how to preserve specimens.

Examination for accurate gender determination has to balance ability to sex specimens in a laboratory without histological sectioning facilities, with the need to retain some or many specimens for later species identifications and gonad microscopy. It also has to balance the need for standardized penis length measures using live or preserved specimens. Campbell (1990) has shown that the penis remained contracted in live and formalin-preserved specimens of *Nucella canaliculata*, while a freezing technique provided more accurate penis length measures. The formalin-preserved specimens of *Thais luteostoma* show that some penes were preserved in an extended form, increasing variability in penis length measures (see Figure 5.2).

The appropriate protocol appears to be to examine all specimens alive by the two-step gender determination procedure, i.e., the examination of secondary sexual characters and the gonad squashes. Avoid contact with the penis and measure it consistently (standardize the penis length measure). This can be applied in the field or in a laboratory without sectioning facilities. Otherwise, if necessary, the undeterminable specimens can be preserved for further gonad sectioning. Specimens for taxonomic purposes should be preserved intact. If the latter specimens are to be used for imposex studies after species confirmation, then a preservation technique relaxing the tissues rather than contracting them should be employed.

Figure 5.2 **Scattergram showing variability in male penis length of *Thais luteostoma*** [L = live specimens; P = formalin-preserved specimens; the outliers shown are the preserved penes with extended penial flagella]



## 5.3

## Species Identifications

The species investigated was first identified as *Thais kieneri* (Deshayes, 1844) using restricted identification resources in the field. Subsequently, it was identified by authorities as *T. clavigera* (Kuster, 1858) and then *T. luteostoma* (Holten, 1803) (*T. kieneri* is a junior synonym).

The species is widely distributed in Ambon Bay, occupying hard surface habitats such as rocks, concrete and wooden structures. Most specimens collected from harbours were overgrown by other organisms (see Figure 2.5). Biocorrosion caused by these organisms blunts the shell sculpture, even often damages it, resulting in dimensions and form different from the clean shells. How big the difference is depends on how heavily the shell is fouled and probably the type of fouling organism. This, however, should not cause difficulties in identification since the ventral part of the shell usually remains clean and can be used for comparison.

Another prominent feature noticed in this species is that the specimens collected under docks generally had short blunt spines on the shells. Specimens collected from 'clean' areas such as Amahusu Shore (rocky habitat) had prominent spines and ribs. Shell variation of similar type is also reported for other neogastropods. For example, *Nucella lamellosa* from the northeast Pacific has two forms of shells, i.e., a fluted, highly sculptured type and a smooth type (Carefoot, 1977).

*Thais clavigera* is similar to *T. luteostoma* (Taylor, 1977). Its taxonomic history is complicated (Abe, 1985). This species is a common intertidal thaidid with a geographical distribution from Japan to southern China (Abe, 1985), with the southern fringe of distribution in Hong Kong (Abe, 1983). *T. clavigera* was reported from Japan (Abe, 1985; Fujioka, 1985) and Hong Kong (Taylor, 1977; Abe, 1983).

It also had been reported from the west coast of the Malay Peninsula (Way and Purchon, 1981).

*Thais luteostoma* is another common intertidal thaidid, but has a wider distribution, i.e., from Japan to Australia (Abbott and Dance, 1982). The species was reported from Japan (Fujioka, 1985), Hong Kong (Taylor, 1977), Malaysia (Ellis and Pattisina, 1990), Papua New Guinea (Hinton, 1972) and tropical Australia (Wilson and Gillett, 1971; Shaw, 1980).

The geographical distributions of *Thais clavigera* and *T. luteostoma* are mapped in Figure 5.3. Although the two species overlap in distribution from Japan to Malaysia, they occupy different intertidal habitats; *T. clavigera* is common in the mid-littoral zone, while *T. luteostoma* is common in the lower mid-littoral (Taylor, 1977).

Although the biology of *Thais luteostoma* in Ambon is not known, the species is reported from neighbouring regions, i.e., Papua New Guinea (Hinton, 1972) and tropical Australia (Wilson and Gillett, 1971). These references were not available in the field when the preliminary survey was conducted. The only available illustration matching the description of the selected specimen was found in Shaw (1980), which uses the name *T. kieneri*.

Wells (1990) has indicated strong affinities between Indonesian, New Guinean and northern Australian shallow-water marine neogastropods. Many other neogastropod species expected in the Ambon area are listed in several references, e.g., Hinton (1972), Wilson and Gillett (1971) and Wells (1980). By comparing them with the list of imposex neogastropod species in Appendix 1, it appears that tropical genera of families Muricidae and Nassariidae are very likely to be important for imposex investigation in the Ambon area, as they include most of the tropical neogastropod species (Wells, 1980, 1990). In general the species selected should occur exposed on rocky shores and other hard surfaces, at low to mid-tide

levels. They should be relatively large (2-3 cm shell length), abundant and collectable, if possible, by a randomization procedure.

#### 5.4 **The Cause of Imposex in Ambon Bay**

These surveys show that imposex is widespread in Ambon Bay, but with localized sites of high incidence and intensity. The two sites with highest intensity are Galala Tuna Harbour (1988 and 1989) and Yos Soedarso (1988) (see Figure 5.4). This is a distributional pattern of 'hotspots' imposed on low ambient levels matching that elsewhere. Imposex is most extreme in areas of high vessel activity, in terms of traffic, dock and maintenance facilities.

The surveys of antifouling paint use showed that TBT was used rarely in Ambon Harbour, with the slipway at Galala Tuna Harbour being the only location where such paints were found.

In conclusion, there are reasonable grounds for concluding that the neogastropod imposex in Ambon Bay may be induced by TBT-based paints. These paints may come from occasional local use, but are more likely derived from leaching off deep-sea vessels, particularly foreign vessels docking at the two harbours.

Figure 5.3 **Geographical distribution of *Thais clavigera* (Kuster) and *T. luteostoma* (Holten)** [square = *T. clavigera*; circle = *T. luteostoma*; 1 = Fujioka, 1985; 2 = Abe, 1985; 3 = Taylor, 1977; 4 = Abe, 1983; 5 = Way & Purchon, 1981; 6 = Ellis & Pattisina, 1990; 7 = Hinton, 1972; 8 = Shaw, 1980; 9 = Wilson & Gillett, 1971; base map is the courtesy of Hinton, 1972]

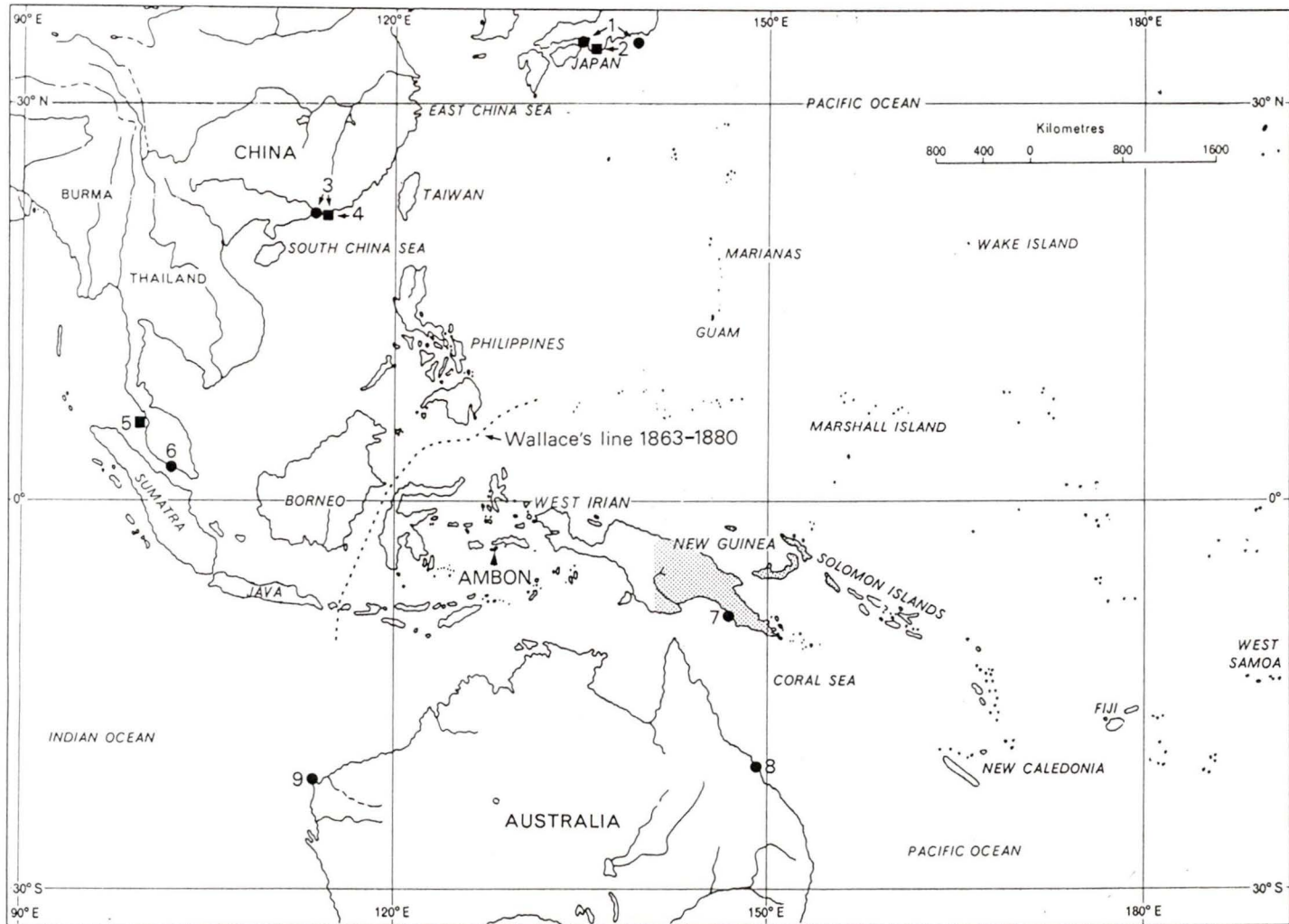
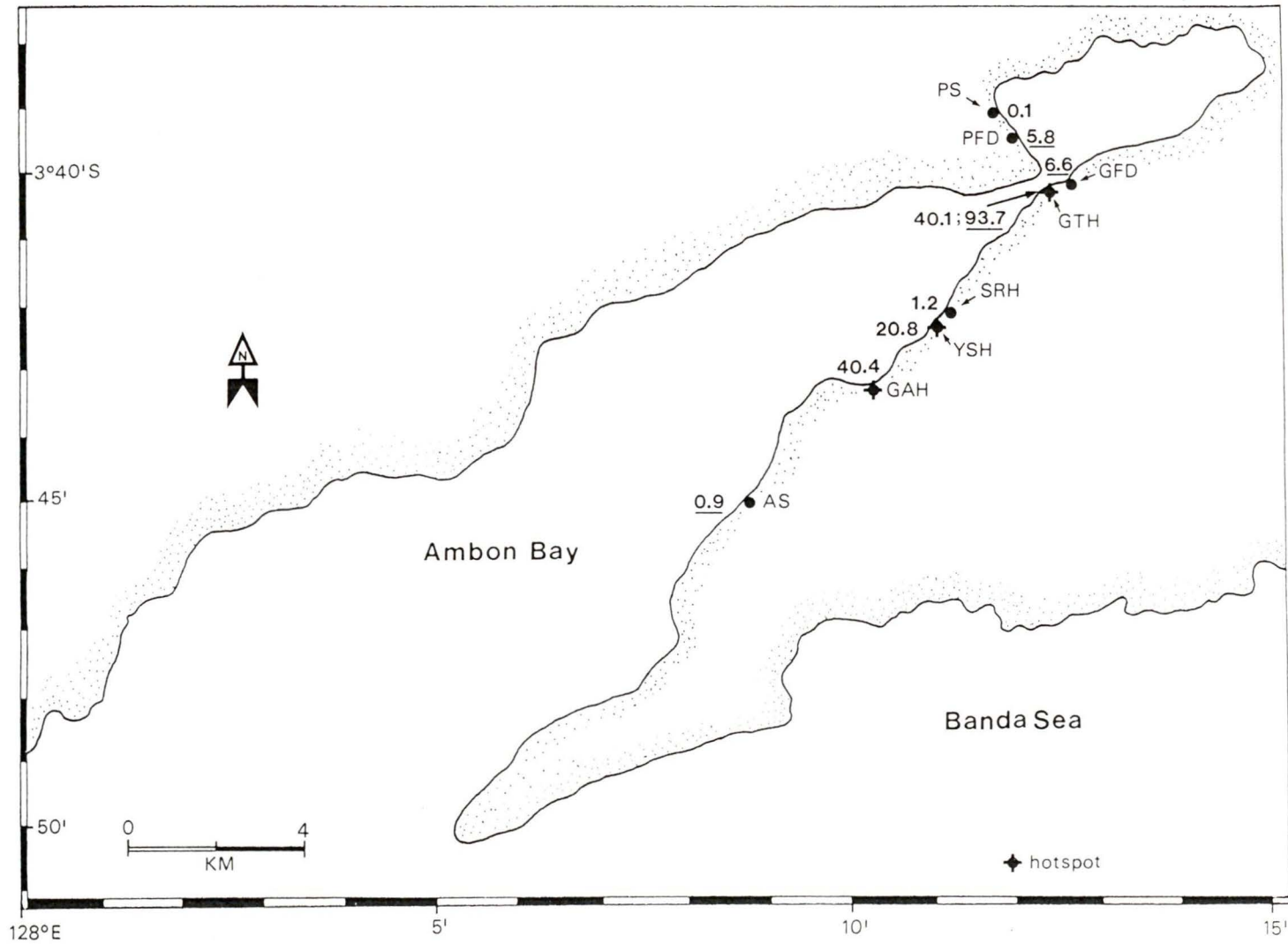


Figure 5.4 **Distribution of imposex intensity in Ambon Bay** [AS = Amahusu Shore; GAH = Gudang Arang Harbour; YSH = Yos Soedarso Harbour; SRH = Slamet Riyadi Harbour; GTH = Galala Tuna Harbour; GFD = Galala Ferry Dock; PFD = Poka Ferry Dock; PS = Poka Shore; the figures shown are the  $RPS_{pop}$  values, those without underlines are the 1988 result, while the underlined ones are the 1989 result]



## 5.5

**Summary of Conclusions**

1. Neogastropod imposex occurred in the tropical harbour of Ambon, remote from major traffic routes.
2. The pattern of imposex distribution consisted of 'hotspots' localized at docks catering to high seas traffic (freighters and tuna fish boats), plus a more widely spread low ambient level.
3. The biocide tributyltin, a known cause for neogastropod imposex, and mortalities in oyster and mussel farms, is rarely used in the Ambon area due to its higher cost compared to other antifouling paints. However, commercial deep sea ships in the harbours can be expected to use it.
4. *Thais luteostoma* was the most suitable species for imposex determinations in Ambon Harbour due to its relatively large size (2-3 cm shell length), availability at mid-tide to sub-tidal levels, in moderate numbers so that 20-50 specimens could be collected. *T. luteostoma* was available to divers collecting from dock pilings at low tide. These characteristics for species selection should apply in surveys elsewhere.
5. Imposex measures consist of presence-absence records and penis length. Some indices have been derived from these measures for mapping and graphical comparisons. They include relative penis size (female vs male) and the relationship between relative penis size and relative frequency. The penis length measure needs to be obtained in such a way that cubing the measure is an adequate index of penis bulk. The nature of this measure will vary with penis form. It can be the diameter of a recurved penis or along the length of an extended penis. Consistency must be achieved for any species.
6. In Ambon Bay, harbour sites and some others showed 100% incidence of imposex, including a site about 4 km from the main harbour. However,

Relative Penis Size varied from less than 1% to almost 100%, i.e., the size of the female penis ranged from being only just measurable to about that of the male penis.

## 5.6

**Recommendations**

1. Wide distribution of *Thais luteostoma* in Ambon Bay, but at low to moderate abundance at separated sites and at diverse habitats, suggests that recruitment to the local populations (enclaves) is probably by settling larvae. This is a good indication of a suitable bioindicator since larval influx will maintain species availability for long-term monitoring purposes. Studies on the biological and ecological aspects of this species should be conducted locally to fill the present gap in our knowledge.
2. Other neogastropod species that have potential as bioindicators, e.g., *Nassarius* and *Mancinella*, are found in Ambon Bay. They should be examined for a comparative imposex study. *Nassarius* are generally infaunal (inhabit muddy sediment to several cm depth) and can be used to monitor sediment conditions.
3. Chemical analysis of TBT is slow and requires expensive equipment. The analyses are not practical for broad-scale geographic surveys requiring many samples. Nevertheless, chemical analyses should be used to confirm the biological indications that the two harbours are sources of TBT contamination. The analytical methods employed should allow the analyses of tin (Sn) in the bioavailable forms. Alternatively, other biological methods, such as field transplantation by exchanging specimens between 'clean' and 'hotspot' areas, or by using 'clean' specimens of other types of molluscs known to be affected (e.g., oysters) would provide additional comparative

information. Chemical analyses of the other heavy metals should also be conducted for comparisons.

4. Water mass exchanges in Ambon Bay depend on the monsoon. Poor water exchange occurs during the West Monsoon (Dry Season). The most severe impact of TBT contamination in the bay has probably occurred during this period of the year (October to March). Long-term field monitoring of imposex occurrence in the bay by transplantation methods should be conducted to obtain information about seasonal patterns of induction. This would indicate any seasonal variation in TBT contamination.
5. TBT is the cause of the oyster industry decline in France. Some oyster farms in Maluku (Moluccas) Province, e.g., pearl oyster farms, are visited by foreign ships. The occurrence of neogastropod imposex in Ambon Bay gives an early warning of possible TBT-antifouling paint contamination at such farm sites. TBT contamination surveys should be made at any bivalve mollusc farm where neogastropod imposex can be demonstrated.

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**Appendices**

## Appendix 1 A list of neogastropod imposex-species

## Order NEOGASTROPODA Thiele, 1929 (= Stenoglossa Bouvier, 1887)

## I. Superfamily Muricoidea Rafinesque, 1815 (= Rachiglossa Gray, 1853)

1<sup>0</sup> Family Muricidae Rafinesque, 1815

1. *Calotrophon ostrearum* (Conrad, 1846)<sup>1)</sup>  
[Synonyms: *C. floridana* (Conrad, 1869), *C. attenuata* (Dall, 1890), *C. perplexus* (Olsson & Harbison, 1935)<sup>a)</sup>; *Murex ostrearum* Conrad, 1846, *Urosalpinx floridana* Conrad, 1869<sup>b)</sup>]
2. *Ceratostoma foliatum* (Gmelin, 1791)<sup>2)</sup>  
[Synonyms: *C. foliatum* Martyn, *Purpura foliatum* Martyn<sup>a)</sup>; *Murex foliatus* Gmelin, 1791<sup>b)</sup>]
3. *Cronia margariticola* (Broderip, 1833)<sup>3)</sup>  
[Synonyms: *C. thiarella* Quoy & Gaimard, 1833, *C. rhyssa* Dall, 1932<sup>c)</sup>; *Murex undatus* Chemnitz, 1795, *Purpura muricoides* Blainville, 1832<sup>d)</sup>]
4. *Drupella rugosa* (Born, 1778)<sup>3)</sup>  
[Synonyms: *D. concatenatus* Lamarck, 1822, *D. fragum* Blainville, 1832<sup>c)</sup>]
5. *Eupleura caudata* (Say, 1822)<sup>1)</sup>  
[Synonym: *Ranella caudata* Say, 1822<sup>b)</sup>]
6. *E. caudata eterae* B. Baker, 1951<sup>4)</sup>
7. *E. sulcidentata* Dall, 1890<sup>1)</sup>  
[Synonym: *E. caudata sulcidentata* Dall, 1890<sup>b)</sup>]
8. *Haustrum haustorium*<sup>5)</sup>  
[Synonym: *Purpura haustorium* (Gmelin, 1791)<sup>e)</sup>]
9. *Lepsiella albomarginata*<sup>6)</sup>
10. *L. scobina* Quoy & Gaimard, 1833<sup>6)</sup>
11. *Morula marginalba* Blainville, 1832<sup>7)</sup>
12. *M. musiva* (Kiener, 1835)<sup>3)</sup>
13. *Murex florifer dilectus* A. Adams, 1855<sup>1)</sup>  
[Synonym: *M. arenarius* Clench & Farfante, 1945 (non Steuer, 1912)<sup>a)</sup>]
14. *M. pomum* Gmelin, 1791<sup>1)</sup>  
[Synonyms: *M. asperrimus* Lamarck, 1822, *M. mexicanus* Petit, 1852, *M. pomiformis* Morch, 1852, *M. oculatus* Reeve, 1845<sup>a)</sup>]
15. *Nucella canaliculata* (Duclos, 1832)<sup>8)</sup>  
[Synonyms: *Thais canaliculata*<sup>f)</sup>; *Nucella analoga* Forbes, 1850, *N. decemcostata* Middendorff, 1849<sup>a)</sup>]

16. *N. emarginata* (Deshayes, 1839)<sup>9,8)</sup>  
 [Synonyms: *Thais emarginata*<sup>f)</sup>; *Nucella conradi* Reeve, 1846, *N. ostrina* Gould, 1852<sup>a)</sup>]
17. *N. lamellosa* (Gmelin, 1791)<sup>8)</sup>  
 [Synonyms: *Thais lamellosa*<sup>f)</sup>; *Purpura crispata* Martyn, *Nucella rugosus* Perry, 1811, *N. ferrugineus* Eschscholtz, 1829, *N. lactuca* Eschscholtz, 1829, *N. septentrionalis* Reeve, 1846, *N. plicata* von Martens, 1872, *N. quillayutea* Reagan, 1909<sup>a)</sup>]
18. *N. lapillus* (Linnaeus, 1758)<sup>10,11,12)</sup>  
 [Synonyms: *Thais lapillus*, *Buccinum lapillus* Linnaeus, 1758, *Purpura lapillus* (Linnaeus, 1758)<sup>g)</sup>]
19. *N. lima* (Gmelin, 1791)<sup>1,13)</sup>  
 [Synonyms: *N. saxicola* Valenciennes, 1846, *N. attenuata* Reeve, 1846<sup>a)</sup>]
20. *Ocenebra erinacea* (Linnaeus, 1758)<sup>14,15,16)</sup>  
 [Synonyms: *Murex torosus* Lamarck, 1816, *M. tarentinus* Lamarck, 1822<sup>b)</sup>; *Murex erinaceus* Linnaeus, 1758<sup>b,g)</sup>; *Tritonalia erinacea* (Linnaeus, 1758), *Ceratostoma erinaceum* (Linnaeus, 1758)<sup>g)</sup>]
21. *O. lurida* (Middendorff, 1848)<sup>8)</sup>  
 [Synonyms: *Tritonium (Fusus) luridum* Middendorff, 1848, *Vitularia asper* Baird, 1863, *Ocenebra (lurida) munda* Carpenter, 1864, *Tritonalia sclera* Dall, 1919<sup>b)</sup>]
22. *Thais clavigera* (Kuster, 1858)<sup>17)</sup>  
 [Synonyms: *T. problematica* Baker, 1891<sup>d)</sup>; *Purpura clavigera* Kuster, 1858, *P. tumulosa* Reeve, 1846<sup>h)</sup>]
23. *T. haemastoma* (Linnaeus, 1767)<sup>18)</sup>
24. *T. haemastoma canaliculata* (Gray, 1839)<sup>1)</sup>  
 [Synonym: *T. haysae* Clench, 1927<sup>a)</sup>]
25. *T. haemastoma floridana* (Conrad, 1837)<sup>1)</sup>
26. *T. luteostoma* (Holten, 1803)<sup>19)</sup>  
 [Synonyms: *T. kieneri* (Deshayes, 1844), *T. pica* Blainville<sup>1)</sup>]
27. *T. orbita*<sup>5)</sup>
28. *Urosalpinx cinerea* (Say, 1822)<sup>1)</sup>  
 [Synonym: *Fusus cinereus* Say, 1822<sup>b,g)</sup>]
29. *U. cinerea follyensis* B. Baker, 1951<sup>4)</sup>
30. *U. perrugata* (Conrad, 1846)<sup>1)</sup>  
 [Synonym: *Fusus perrugatus* Conrad, 1846<sup>b)</sup>]
31. *U. tampaensis* (Conrad, 1846)<sup>1)</sup>  
 [Synonym: *Murex tampaensis* Conrad, 1846<sup>b)</sup>]

2<sup>0</sup> Family **Buccinidae** Rafinesque, 1815

32. *Colus halli* (Dall, 1873)<sup>8)</sup>  
 [Synonyms: *C. jordani* Dall, 1913, *C. dalmasius* (Dall, 1919); probable synonyms: *C. erroneus* Dall, 1919, *C. georgianus* Dall, 1920, *C. halimeris* Dall, 1919, *C. herendeeni* Dall, 1902, *C. mordita* Dall, 1919, *C. tahwitanus* Dall, 1919<sup>j)</sup>]

33. *Neptunea phoenicea* (Dall, 1891)<sup>8)</sup>

[**Synonyms:** *N. lyrata lyrata* (Gmelin, 1791), *Chrysodomus lirata* Martyn, *Cymatium pacificum* Dall, 1909, *N. obsoleta* Golikov, 1963<sup>a)</sup>]

34. *Pisania tinctus*<sup>1)</sup>

[**Synonyms:** *P. tincta* (Conrad, 1846), *Tritonidea bermudensis* Dall & Simpson, 1901<sup>a)</sup>]

35. *Searlesia dira* (Reeve, 1846)<sup>8)</sup>

3<sup>0</sup> Family **Nassariidae** Iredale, 1916

36. *Bullia rhodostoma*<sup>20)</sup>

37. *Hinia reticulata* (Linnaeus, 1758)<sup>21,22,23)\*</sup>

[**Synonyms:** *Nassarius reticulatus* (Linnaeus, 1758), *Buccinum reticulatum* Linnaeus, 1758, *Nassa reticulata* (Linnaeus, 1758), *Arcularia reticulata* (Linnaeus, 1758), *Hima reticulata* (Linnaeus, 1758)<sup>g)</sup>]

\*Bryan *et al.* (1986) and Tallmark *in* Laughlin and Linden (1987) listed it as *Nassarius reticulatus*.

38. *Ilyanassa obsoleta* Say, 1822<sup>24,25)\*</sup>

[**Synonym:** *Nassarius obsoletus* (Say, 1822)<sup>k)</sup>]

\*Smith (1971, 1980, 1981a-c) listed it as *Nassarius obsoletus*.

39. *Nassarius coronatus* (Bruguier, 1789)<sup>26)</sup>

[**Synonyms:** *N. bronni* Philippi, 1849 (non Anton, 1839), *N. fasciolatus* of Hirase, 1936 and Cotton, 1955<sup>e)</sup>]

40. *N. dorsatus* (Roeding, 1798)<sup>27)</sup>

[**Synonyms:** *N. trifasciatum* Gmelin, 1791, *N. livida* Gray in King, 1827, *N. canaliculatum* Lamarck, 1822, *N. unicolorum* Kiener, 1834, *N. badia* A. Adams, 1852, *N. unicolorata* Reeve, 1853, *N. rutilans*, Reeve, 1853<sup>e)</sup>]

41. *N. luridus* (Gould, 1849)<sup>26)</sup>

[**Synonyms:** *N. punctata* A. Adams, 1852 (non Kiener, 1834), *N. lentiginosa* A. Adams, 1852, *N. dispar* A. Adams, 1852<sup>e)</sup>]

42. *N. pullus* (Linnaeus, 1758)<sup>26)</sup>

[**Synonyms:** *N. thersier* Bruguier, 1789, *N. bimaculosa* A. Adams, 1852, *N. gracilis* Pease, 1868, *N. acypha* von Martens, 1887<sup>e)</sup>]

43. *N. trivittatus* (Say, 1822)<sup>1)</sup>

44. *N. vibex* (Say, 1822)<sup>1)</sup>

4<sup>0</sup> Family **Columbellidae** Swainson, 1840

45. *Anachis avara* (Say, 1822)<sup>1)</sup>

[**Synonym:** *A. cleta* Duclos, 1846<sup>a)</sup>]

46. *Mitrella lunata* (Say, 1826)<sup>1)</sup>

[**Probable synonyms:** *M. duclosiana* Orbigny, 1842, *M. spirantha* Ravenel, 1861, *M. gouldiana* Stimpson, 1851, *M. wheatleyi* DeKay, 1843, *M. dissimilis* Stimpson, 1851, *M. zonalis* Gould, 1848<sup>a)</sup>]

5<sup>0</sup> Family **Melongenidae** Gill, 187147. *Busycon carica* (Gmelin, 1791)<sup>1)</sup>[Synonyms: *B. aruanum* Hollister, 1958 (non Linnaeus, 1758), *B. muricatum* Roding, 1758<sup>a)</sup>]48. *B. contrarium* (Conrad, 1840)<sup>1)</sup>49. *Melongena corona* (Gmelin, 1791)<sup>1)</sup>[Synonyms: *M. aspinosa* Dall, 1890, *M. belknapi* Petit, 1852, *M. inspinata* Richards, 1933, *M. perspinosa* Pilsbry & Vanatta, 1934, *M. subcoronata* Heilprin, 1887<sup>a)</sup>]6<sup>0</sup> Family **Fasciolaridae** Gray, 185350. *Fasciolaria lilium hunteria* (G. Perry, 1811)<sup>1)</sup>51. *Pleuroploca gigantea* (Kiener, 1840)<sup>1)</sup>7<sup>0</sup> Family **Olividae** Latreille, 182552. *Olivella biplicata* (Sowerby, 1825)<sup>1)</sup>[Synonyms: *O. angelena* T.S. Oldroyd, 1918, *O. fucana* T.S. Oldroyd, 1921<sup>a)</sup>; probable synonym: *O. parva* T.S. Oldroyd, 1921<sup>a)</sup>]8<sup>0</sup> Family **Marginellidae** Fleming, 182853. *Marginella apicina* Menke, 1828<sup>1)</sup>[Synonyms: *M. caribaea* Orbigny, 1842, *M. conoidalis* Kiener, 1841, *M. flavida* Redfield, 1846, *M. livida* Hinds, 1844, *M. virginea* Jousseaume, 1875<sup>a)</sup>]II. Superfamily **Conoidea** Rafinesque, 1815 (= *Taxoglossa* Troschel, 1847)9<sup>0</sup> Family **Conidae** Fleming, 182254. *Conus mediterraneus* Hwass in Bruguiere, 1792<sup>1)</sup>10<sup>0</sup> Family **Terebridae** Morch, 185255. *Terebra dislocata* (Say, 1822)<sup>1)</sup>[Synonyms: *T. petiti* Kiener, 1939, *T. rudis* Gray, 1834<sup>a)</sup>]56. *T. protexta* Conrad, 1845<sup>1)</sup>11<sup>0</sup> Family **Turridae** Swainson, 184057. *Kurtziella cerina* (Kurtz & Stimpson, 1851)<sup>1)</sup>58. *Oenopota turricula* (Montagu, 1803)<sup>1)\*</sup>[Synonyms: *Propebela turricula* (Montagu, 1803), *P. ecarinata* Sars, 1878, *P. scalaris* Moller, 1842, *O. elegans* (Moller, 1842), *Lora rosea* (Sars, 1846), *L. turricula* (Montagu, 1803), *Murex turricula* Montagu, 1803, *Pleurotoma turricula* (Montagu, 1803)<sup>g)</sup>]\*Jenner (1979) listed it as *Propebela turricula*.

Reference:

- 1) Jenner, 1979; 2) Saavedra Alvarez and Ellis, 1990; 3) Ellis and Pattisina, 1990; 4) Griffith and Castagna, 1962; 5) Stewart *et al.*, *in press*; 6) Smith and McVeagh, *in press*; 7) Wilson, 1989; 8) Bright and Ellis, 1990; 9) Houston, 1971; 10) Blaber, 1970; 11) Pondick, 1981; Miller and Pondick, 1984; 12) Bryan *et al.*, 1986, 1987; Gibbs and Bryan, 1986; Gibbs *et al.*, 1987, 1988; Davies *et al.*, 1987; 13) Short *et al.*, 1989; 14) Poli *et al.*, 1971 in Gibbs *et al.*, 1990; 15) Feral, 1976 in Gibbs *et al.*, 1990; 16) Gibbs *et al.*, 1990; 17) Morton, *personal communication*, 1988; 18) Spence *et al.*, 1990; 19) This thesis; 20) Brown, 1982; 21) Bryan *et al.*, 1986; 22) Tallmark in Laughlin and Linden, 1987; 23) Gibbs *et al.*, 1990; 24) Smith, 1971, 1980, 1981a-c; 25) Bryan *et al.*, 1989; 26) Brodie *et al.*, 1989; 27) Mitchell, 1989.
- a) Abbott, 1974; b) Radwin and D'Attilio, 1976; c) Cernohorsky, 1972; d) Fujioka, 1985; e) Dance, 1974; f) Morris *et al.*, 1980; g) Fretter and Graham, 1985; h) Abe, 1985; i) Kool, *personal communication*, 1989; j) Kozloff, 1987; k) Smith, 1981a.

Appendix 2 The vas deferens sequence (VDS) (after Gibbs *et al.*, 1987)

---

Stage	State
1	Development of proximal section of vas deferens, prior to the formation of penis.
2	Appearance of a ridge-shaped penis behind the right tentacle.
3	Small penis formed and development of distal section of vas deferens commencing from the penial base.
4	The two sections of vas deferens fused and penis enlarges to a size of the male's.
5	The blockage of vulva by proliferating vas deferens tissue.
6	Capsule gland contains aborted egg capsules.

---

### Appendix 3 The results of experiments on imposex induction by anti-fouling paints

Antifouling paint	Active agent (concentration, %)	Method of exposure <sup>1</sup>	Duration of exposure (day)	Result	Reference
T755 Red (C.A.Woolsey Paint and Color Co., Inc.)	TBT resinate (18.8)	Exposed to seawater leachates in laboratory	35	+	Smith, 1981a
Alumacide Green (Petit Paint Co.)	TBTO and Paris Green <sup>2</sup> (-)	ibid	35, 75	+	ibid
International, "TBT Antifouling", white	TBT (-)	Shell spire painted and replaced on the shore	measured at 1-2 month interval for 15 months	+	Bryan <i>et al.</i> , 1987
International, "Cruiser", self-polishing, white	TBT (-)	ibid	ibid	+	ibid
Blakes, "Tiger" copolymer, red	TBT (-)	ibid	measured at 2 month interval for 9 months	+	ibid
Blakes, "Tiger" tin-free, red	copper (-)	ibid	ibid	-	ibid
502C Red (Gloucester Paints, Inc.)	cuprous oxide (25.2)	Exposed to seawater leachates in laboratory	35	-	Smith, 1981a
711 Copper (C.A.Woolsey Paint and Color Co., Inc.)	copper-bronze <sup>3</sup> (33)	ibid	35	-	ibid

Legend:

<sup>1</sup>) Concentrations of seawater leachates were not mentioned.

<sup>2</sup>) Paris Green contained lead arsenate.

<sup>3</sup>) Bronze is any of various alloys having a large copper content (The Random House Dictionary of the English Language, 1987, p. 266)

Appendix 4 The results of experiments on imposex induction by organotin compounds

Organotin	Purity (%)	Method of exposure	Concentration	Duration of exposure (day)	Result	Reference
Tributyltin chloride (TBT) <sup>1]</sup>	96	Exposed to seawater leachates	0.11%	57-64	+	Smith, 1981b
Bis(tributyltin) oxide (TBTO) <sup>1]</sup>	96	ibid	0.02-0.03%	ibid	+	ibid
Tributyltin abietate (TBT) <sup>2]</sup>	-	ibid	0.57%	ibid	+	ibid
Butyltin trichloride (MBT) <sup>1]</sup>	95	Injection 2	1 <sup>0</sup>	41, 75	-	Bryan <i>et al.</i> , 1988
Dibutyltin dichloride (DBT) <sup>3]</sup>	96	Exposed to seawater leachates	3 <sup>0</sup>	14	-	ibid
		Injection 1	1 <sup>0</sup>	34, 71, 105	-	
Tributyltin chloride (TBT) <sup>3]</sup>	95-97	Exposed to seawater leachates	3 <sup>0</sup>	14	+	ibid
		Injection 1	1 <sup>0</sup>	34, 70	+	
		Injection 2	2 <sup>0</sup>	41, 76	+	
Tetrabutyltin (TTBT) <sup>3]</sup>	97	Exposed to seawater leachates	3 <sup>0</sup>	14	-	ibid
		Injection 1	1 <sup>0</sup>	34, 71, 105	+	
Tripropyltin chloride (TPrT) <sup>3]</sup>	-	Exposed to seawater leachates	3 <sup>0</sup>	14	+	ibid
		Injection 1	1 <sup>0</sup>	34, 70	+	
		Injection 2	2 <sup>0</sup>	41, 76	-	
Triphenyltin chloride (TPhT) <sup>3]</sup>	95+	Exposed to seawater leachates	3 <sup>0</sup>	14	-	ibid
		Injection 2	2 <sup>0</sup>	41, 76	-	

Legend:

- 1] Aldrich Chemical Co.
- 2] Pfaltz & Bauer.
- 3] Ventron Alpha Products.
- 1<sup>o</sup> 1  $\mu\text{g}$  Sn as 1  $\mu\text{l}$  of an ethanolic solution of the appropriate organotin. Two control groups received injections of 1  $\mu\text{l}$  of ethanol only.
- 2<sup>o</sup> 0.5  $\mu\text{g}$  Sn as 1  $\mu\text{l}$  of an ethanolic solution. The control group received injection of 1  $\mu\text{l}$  of ethanol only.
- 3<sup>o</sup> Initial concentrations of 200  $\text{ng Sn l}^{-1}$  of the appropriate organotin in 0.1 ml of ethanol. The control group was exposed to the addition of 0.1 ml of ethanol only. Experimental seawater contained 1-2  $\text{ng Sn l}^{-1}$  of TBT.

## Appendix 5 Antifouling paint survey questionnaire\*

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### Hardware store

1. Do you sell any antifouling paints ?  
(Have you ever sold any antifouling paints ?)
2. What is the primary agent of the paint ?
3. What concentration is it ?
4. Is the paint an imported product ?  
From what country ?
5. What is the name of the manufacturer ?
6. What kind of antifouling paint is used for wooden boats ?  
What for non-wooden boats, e.g., big ships ?
7. Which antifouling paint is commonly purchased by people ?

### Ship slipway

1. What kinds of antifouling paints do you use for painting ships ?
2. What is the primary agent of the paint ?
3. What concentration is it ?
4. Is the paint an imported product ?  
From what country ?
5. What is the name of the manufacturer ?
6. How long can the paint be effective in ship operation ?
7. How often do you repair and paint ship ?
8. What kinds of ships are they ?  
Where from ?

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\* Asked as appropriate during the interviews in varying forms.



UNIVERSITY OF VICTORIA  
Thesis Research Project in Ambon Bay, Indonesia  
Imposex Worksheet 2 — Notes on specimens

Collection # :	Species :
Location :	Specimen # : (m / f / ?)
Date :	Preserved : yes / no
	Preservative :

	<u>Present (+)</u> Absent (-)	Colour	Sketch
Penis			
Vas Deferens			
Brown Gland			
Gonad			
Other noticeable features			

Appendix 7 Morphometric data for *Thais luteostoma*, 1988

Site/Sex (Number of specimen)	Shell measure (cm)			Penis length (cm)
	length	width	height	
<u>Poka Ferry Dock</u>				
Male (8)	3.2	2.0	1.4	0.50
	2.5	1.7	1.1	0.30
	3.1	2.0	1.3	0.35
	3.0	2.0	1.3	0.35
	2.4	1.6	1.1	0.20
	2.3	1.5	1.1	0.25
	2.6	1.6	1.0	0.10
	1.9	1.2	0.7	0.30
<u>Poka Shore</u>				
Female (13)	3.3	2.1	1.2	0.10
	3.6	2.2	1.3	0.10
	4.2	2.6	1.6	0.00
	3.7	2.3	1.4	0.05
	3.3	2.0	1.2	0.05
	3.2	2.1	1.2	0.05
	2.8	1.8	1.2	0.00
	3.4	2.2	1.5	0.05
	2.7	1.8	1.1	0.00
	3.7	2.3	1.6	0.00
	3.2	2.0	1.3	0.05
	3.4	2.2	1.4	0.00
	3.0	1.8	1.2	0.01
Male (10)	3.9	2.5	1.6	0.45
	3.0	1.9	1.3	0.40
	2.9	1.9	1.4	0.45
	3.7	2.3	1.3	0.45
	3.9	2.6	1.5	0.60
	2.8	1.7	1.1	0.30
	2.9	2.0	1.3	0.40
	2.6	1.7	1.1	0.30
	2.8	1.8	1.0	0.35
	2.4	1.6	1.1	0.30
<u>Galala Tuna Harbour</u>				
Female (11)	3.7	2.4	1.3	0.40
	3.1	2.2	1.4	0.35
	3.8	2.7	1.5	0.45
	3.6	2.4	1.3	0.00
	3.5	2.2	1.4	0.40
	3.5	2.2	1.5	0.20
	3.5	2.3	1.4	0.25
	3.6	2.4	1.4	0.40
	3.8	2.5	1.5	0.35
	3.3	2.0	1.5	0.30

Male (13)	3.1	1.9	1.3	0.30	
	3.5	2.1	1.3	0.50	
	3.5	2.2	1.4	0.40	
	3.6	2.1	1.4	0.55	
	3.4	2.1	1.3	0.40	
	3.4	2.3	1.4	0.45	
	3.2	2.1	1.3	0.50	
	3.5	2.2	1.4	0.50	
	3.2	2.2	1.4	0.30	
	3.6	2.1	1.3	0.50	
	3.5	1.9	1.2	0.40	
	3.2	1.9	1.1	0.20	
	2.9	1.8	1.0	0.30	
	3.2	2.0	1.4	0.45	
<u>Slamet Riyadi Harbour</u>					
Female (17)	4.0	2.6	1.8	0.20	
	4.1	2.6	1.5	0.00	
	4.6	3.3	2.0	0.00	
	2.3	1.7	1.3	0.00	
	2.9	1.8	1.1	0.00	
	3.3	2.1	1.3	0.00	
	4.4	2.2	1.6	0.40	
	3.5	2.4	1.2	0.00	
	3.0	1.9	1.1	0.00	
	3.4	2.3	1.2	0.00	
	3.3	2.2	1.1	0.00	
	3.8	2.4	1.2	0.25	
	3.4	2.2	1.5	0.00	
	3.5	2.0	1.2	0.00	
	4.3	2.5	1.5	0.25	
	3.9	2.6	1.4	0.35	
	3.9	2.5	1.3	0.20	
	Male (15)	3.3	1.9	1.4	0.30
		2.8	1.7	0.9	0.30
		3.3	2.2	1.4	0.50
3.2		2.1	1.4	0.30	
2.9		1.6	1.1	0.50	
2.7		1.6	1.1	0.50	
3.6		2.4	1.1	0.50	
3.3		2.0	1.5	0.50	
2.4		1.8	1.1	0.40	
3.6		2.4	1.5	0.45	
3.6		2.4	1.4	0.35	
3.4		2.1	1.0	0.40	
3.7		2.3	1.0	0.45	
3.6	2.5	1.3	0.50		
3.7	2.4	1.3	0.50		
<u>Yos Soedarso Harbour</u>					
Female (12)	4.1	2.6	1.4	0.25	
	4.1	2.6	1.8	0.30	
	3.5	2.3	1.1	0.30	
	4.1	2.7	1.7	0.30	

	3.5	2.5	1.3	0.20
	3.5	2.3	1.3	0.30
	2.7	1.9	1.1	0.25
	3.4	2.3	1.3	0.30
	2.7	1.9	1.1	0.20
	3.9	2.5	1.6	0.25
	3.3	2.3	1.2	0.35
	3.1	2.0	1.3	0.25
Male (14)	4.5	2.9	1.6	0.60
	3.7	2.5	1.5	0.60
	3.5	2.2	1.4	0.50
	2.8	1.8	1.0	0.45
	2.8	1.9	1.1	0.60
	2.8	1.9	1.1	0.50
	3.4	2.1	1.4	0.50
	3.7	2.3	1.5	0.60
	2.6	1.8	1.1	0.35
	2.6	1.8	1.2	0.20
	2.7	1.7	1.0	0.40
	2.7	1.9	1.2	0.35
	2.9	1.9	1.2	0.40
	2.4	1.8	1.2	0.35
<u>Gudang Arang Harbour</u>				
Female (4)	3.5	2.2	1.4	0.00
	2.7	1.7	1.3	0.25
	2.7	2.0	1.1	0.35
	2.7	1.9	1.1	0.25
Male (4)	2.4	1.6	0.9	0.30
	2.3	1.7	1.1	0.30
	2.5	1.5	0.9	0.30
	2.6	1.8	0.9	0.25

---

Appendix 8 Distribution of imposex in *Thais luteostoma* in Ambon Bay, 1988

Site <sup>1)</sup>	# of specimen m f imposex			RFI (%)	Female penis length (mm)				RPS (%)		VDS		Undetermined gender
					Pop. <sup>2)</sup>		Inc. <sup>3)</sup>		Pop. <sup>2)</sup>	Inc. <sup>3)</sup>	Stage	Index	
					Mean	SD	Mean	SD					
PFD	8	0	0										11
PS	10	13	8	61.5	0.35	0.37	0.58	0.30	0.07	0.30	0-2	1.15	1
GTH	13	11	10	90.9	3.09	1.26	3.40	0.77	40.08	53.34	1-4	3.33	4
SRH	15	17	6	35.3	0.97	1.43	2.75	0.82	1.15	26.16	0-4	0.88	3
YSH	14	12	12	100.0	(2.71 0.45) <sup>4)</sup>				(20.79) <sup>4)</sup>		2-4	3.82	
GAH	4	4	3	75.0	2.13	1.49	2.83	0.58	40.38	95.71	0-4	2.00	

Legend:

- 1) PFD = Poka Ferry Dock;  
PS = Poka Shore;  
GTH = Galala Tuna Harbour;  
SRH = Slamet Riyadi Harbour;  
YSH = Yos Soedarso Harbour;  
GAH = Gudang Arang Harbour.
- 2) Pop. = population, i.e including normal females (females with zero penis length).
- 3) Inc. = incidence, i.e., excluding normal females.
- 4) At RFI = 100%, population and incidence parameters are identical.

## Appendix 9 Results of normality and homoscedasticity tests for female penis length, 1988

### I. Summary statistics of female penis length

Statistic	Site <sup>1)</sup>				
	PS	GTH	SRH	YSH	GAH
Sample size (n)	13	11	17	12	4
Minimum value	0	0	0	0.20	0
Maximum value	0.10	0.45	0.40	0.35	0.35
Range	0.10	0.45	0.40	0.15	0.35
Mean	0.035	0.309	0.097	0.271	0.213
Variance ( $\sigma^2$ )	0.001	0.016	0.020	0.002	0.022
Standard Deviation	0.037	0.126	0.143	0.045	0.149
Coefficient of Skewness (G1)	0.549	-1.351	0.952	-0.132	-0.797
Coefficient of Kurtosis (G2)	-0.885	1.297	-0.650	-0.730	-0.853
Coefficient of Variation	1.042	0.408	1.474	0.166	0.703

1) PS = Poka Shore; GTH = Galala Tuna Harbour; SRH = Slamet Riyadi Harbour; YSH = Yos Soedarso Harbour; GAH = Gudang Arang Harbour.

### II. Lilliefors Test for normality

#### 1. Hypothesis:

$H_0$ : Penis lengths are normally distributed.

#### 2. Level of significant:

$\alpha = 0.05$

#### 3. Criteria:

Reject  $H_0$  if  $P \leq 0.05$

#### 4. Result:

#### 5. Conclusion:

Site	n	$d_{\max}$	P	
PS	13	0.216	0.099	Accept $H_0$ (normal)
GTH	11	0.199	0.279	Accept $H_0$ (normal)
SRH	17	0.398	0.000	Reject $H_0$ (non-normal)
YSH	12	0.241	0.052	Accept $H_0$ (normal)
GAH	4	0.349	0.102	Accept $H_0$ (normal)

[ $d_{\max}$  = the absolute value of the maximum difference between the relative cumulative frequencies].

### III. Bartlett Test for homoscedasticity

#### 1. Hypothesis:

$$H_0: \sigma^2_{PS} = \sigma^2_{GTH} = \sigma^2_{SRH} = \sigma^2_{YSH} = \sigma^2_{GAH}$$

#### 2. Level of significance:

$$\alpha = 0.05$$

#### 3. Criteria:

Reject  $H_0$  if  $P \leq 0.05$

#### 4. Result:

$$\chi^2 = 28.204, \quad DF = 4, \quad P = 0.000$$

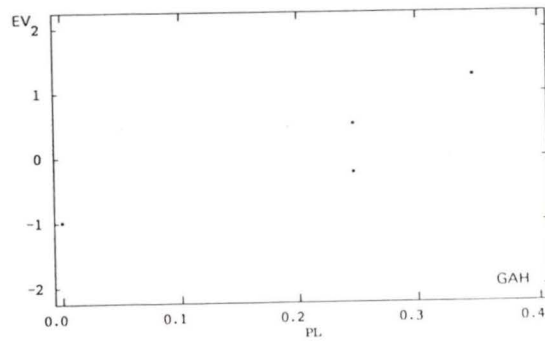
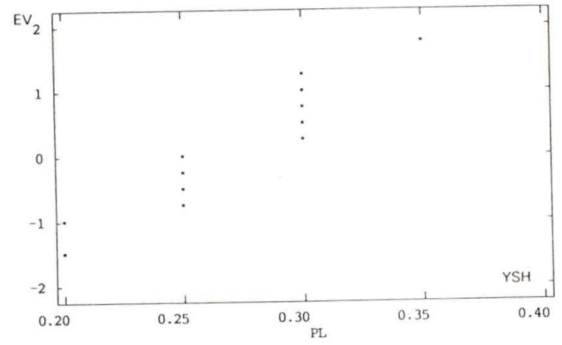
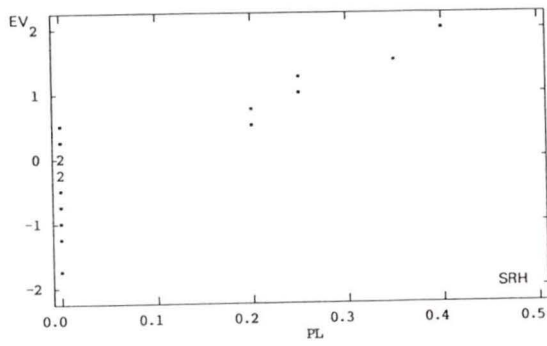
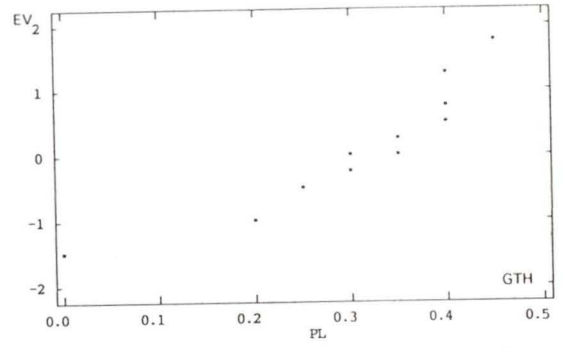
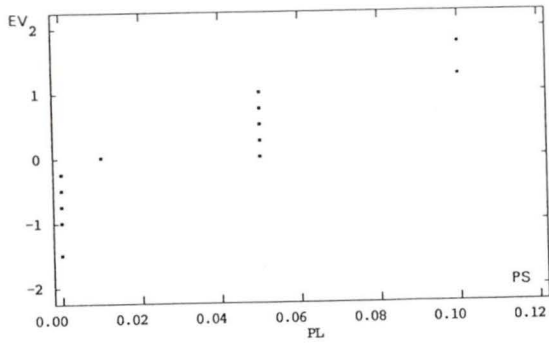
[DF = degree of freedom; P = probability].

#### 5. Conclusion:

Reject  $H_0 \Rightarrow$  The variances of penis length of the five sites are heterogeneous.

### IV. Normal Probability Plot

[see page 130; EV = expected value, PL = penis length (cm)]



## Appendix 10 Antifouling paints used locally in Ambon

I. Some Antifouling Paints Sold in Ambon City<sup>1)</sup>

Name of paint	Manufacturer	Active agent (concentration) <sup>2)</sup>	Hardware store
Transocean Marine Paint	Nippon Paint Co., Ltd. Indonesia	Cuprous oxide (NA)	Guna Rakyat
Nippon Paint	ibid	Cuprous oxide (NA)	Sama Lagi
Nippon Marine Coating, Nippelux Copper Anti Fouling	ibid	Cuprous oxide (NA)	Angin Timur; Rekayasa
Nippon Marine Coating, Nippelux Tropical Anti Fouling	ibid	Not listed	Rekayasa

II. Some antifouling paints used in the Galala Slipway, Ambon<sup>3)</sup>

Name of paint	Manufacturer	Active agent (concentration) <sup>2)</sup>
Transtropic Super Anti Fouling	P.T. Nippon Indonesia Co., Ltd. P.T. United Transocean Marine Paint Co., Ltd.	Cuprous oxide (NA)
Transtropic Anti Fouling	P.T. Nippon Indonesia Co., Ltd.	Cuprous oxide (NA)
AF-Seaflor SP	Chugoku Marine Paints, Ltd.	Triphenyltin (NA) <b>Tributyltin</b> (NA) Cuprous oxide (NA)

1) data collected on August 10, 1988.

2) NA = Not Available.

3) data collected on August 30, 1988.

Appendix 11 Derivation of the relationship between RFI, Population and Incidence RPS indices

Imposex frequency (Relative Frequency of Imposex, RFI) is the simplest imposex measure. It is defined as follows:

$$RFI = \frac{\text{number of imposex females}}{\text{total number of females}} \dots\dots\dots(1)$$

At very high TBT-contaminated sites, usually all females have been affected or RFI = 100%. This means that all females are imposex females, or the number of imposex females equals to the total number of females.

$$RFI = \frac{\text{number of imposex females}}{\text{total number of females}} = 1, \text{ or}$$

$$\# \text{ imposex females} = \# \text{ all females}$$

Therefore, the sum of penis lengths of the imposex females equals to that of all the females. Thus,

$$\begin{aligned} \Sigma L_{\text{imposex females}} &= \Sigma L_{\text{all females}}, \text{ or} \\ \Sigma L_{\text{inc}} &= \Sigma L_{\text{pop}} = \Sigma L, \end{aligned}$$

- where: L = penis length;
- inc = incidence (refers to the affected or imposex females only);
- pop = population (refers to the affected or imposex females *and* normal females)

If both numerator and denominator of Equation (1) are multiplied by  $\Sigma L$ , the results are as follows:

$$\begin{aligned}
 \text{RFI} &= \frac{\# \text{ imposex females} \cdot \Sigma L}{\# \text{ total females} \cdot \Sigma L} \\
 \Rightarrow \text{RFI} &= \frac{\Sigma L / \# \text{ total females}}{\Sigma L / \# \text{ imposex females}} \\
 \Rightarrow \text{RFI} &= \frac{\bar{L} \text{ all females}}{\bar{L} \text{ imposex females}}, \dots\dots\dots(2)
 \end{aligned}$$

where:  $\bar{L}$  is mean penis length.

Since Bryan *et al.* (1986) defined the Relative Penis Size (RPS) as: (mean female penis length)<sup>3</sup> / (mean male penis length)<sup>3</sup>, the RFI can be related to the RPS by modifying Equation (2) as follows:

$$\begin{aligned}
 \text{RFI} &= \frac{\{(\bar{L} \text{ all females} / \bar{L} \text{ males})^3\}^{1/3}}{\{(\bar{L} \text{ imposex females} / \bar{L} \text{ males})^3\}^{1/3}}, \text{ or} \\
 \text{RFI} &= \left[ \frac{\text{RPS}_{\text{pop}}}{\text{RPS}_{\text{inc}}} \right]^{1/3}, \dots\dots\dots(3)
 \end{aligned}$$

where:  $\text{RPS}_{\text{pop}}$  is the Population RPS, i.e., RPS of all the females in a population (= Bryan's RPS);  
 $\text{RPS}_{\text{inc}}$  is the Incidence RPS, i.e., RPS of the affected or imposex females only.

Appendix 12 Distribution and abundance of *Thais luteostoma* at the sampling sites, 1989

Date (May)	Site <sup>1)</sup>	Type of Habitat	Type of collection	Specimen <sup>2)</sup>	Number of specimen			
					Total	f	m	?
19	PFD	Wood	Hand	L	10	2	8	0
				P	28	10	17	1
25	GFD	Rock	Hand	L	10	1	5	4
				P	16	8	7	1
26	GTH	Wood	Diving	L	10	1	7	2
				P	25	6	16	3
23	AS	Rock	Hand	L	10	2	6	2
				P	38	11	11	16

Legend: 1) PFD = Poka Ferry Dock;  
 GFD = Galala Ferry Dock;  
 GTH = Galala Tuna Harbour;  
 AS = Amahusu Shore.  
 2) L = Live-examined;  
 P = Preserved in 4% formaldehyde.

Appendix 13 Morphometric data for *Thais luteostoma*, 1989

Location/ Sex (Number of specimen)	Preserved specimen*			Sex (Number of specimen)	Live specimen*			
	SL	SW	PL		SL	SW	PL	
<u>Poka Ferry Dock</u>								
Female (10)	5.0	3.1	0.45	Female (2)	4.8	2.2	0.20	
	3.6	2.1	0.20		4.6	2.6	0.15	
Male (17)	3.1	2.0	0.20	Male (8)	4.1	1.8	0.40	
	2.9	1.9	0.20		4.0	1.9	0.45	
	3.5	2.1	0.20		4.4	1.8	0.50	
	3.1	1.8	0.15		4.3	1.7	0.50	
	3.8	2.4	0.20		4.2	1.8	0.50	
	3.4	2.2	0.30		4.4	1.9	0.40	
	3.5	2.2	0.15		4.0	2.0	0.40	
	3.3	2.0	0.15		3.8	1.4	0.35	
	3.5	2.2	0.50					
	3.0	2.1	0.50					
	3.1	2.0	0.60					
	3.4	2.2	0.50					
	3.4	2.2	0.55					
	3.0	1.8	0.65					
	3.6	2.1	0.80					
3.7	2.3	0.30						
3.9	2.3	0.75						
3.6	2.2	0.50						
3.9	2.3	1.50						
3.5	2.2	0.85						
3.4	2.2	0.45						
3.4	2.4	0.50						
3.5	2.1	0.45						
3.0	1.9	0.70						
3.2	1.9	0.50						
<u>Galala Ferry Dock</u>								
Female (8)	3.9	2.6	0.20	Female (1)	3.5	1.6	0.10	
	3.3	2.1	0.10	Male (5)	3.2	1.6	0.30	
Male (7)	4.0	2.4	0.15		2.9	1.4	0.30	
	3.6	2.4	0.10		3.0	1.5	0.30	
	3.4	2.2	0.10		3.2	1.4	0.25	
	3.5	2.4	0.25		2.9	1.3	0.10	
	3.9	2.6	0.15					
	3.3	2.1	0.15					
	3.6	2.2	0.45					
	3.5	2.2	0.30					
	3.3	2.3	0.45					
	3.3	2.1	0.30					
3.1	2.0	0.40						
3.2	2.0	0.30						
3.3	2.1	0.40						

Galala Tuna Harbour

Female (6)	3.7	2.3	0.35	Female (1)	3.1	1.5	0.30	
	3.5	2.1	0.30		Male (7)	3.2	1.4	0.30
	3.7	2.3	0.30			3.7	1.6	0.45
Male (16)	3.6	2.2	0.23		3.4	1.4	0.45	
	3.7	2.1	0.40		3.2	1.5	0.30	
	3.5	2.3	0.35		2.8	1.4	0.30	
	3.4	2.1	0.25		3.5	1.5	0.30	
	3.4	2.2	0.43		3.0	1.5	0.40	
	2.9	1.8	0.35					
	3.2	2.1	0.35					
	3.8	2.3	0.33					
	3.7	2.2	0.35					
	3.1	1.9	0.40					
	3.0	1.9	0.30					
	2.5	1.7	0.25					
	2.2	1.6	0.30					
	2.5	1.6	0.30					
	2.1	1.4	0.35					
	3.0	2.0	0.45					
2.6	1.7	0.25						
2.9	1.9	0.30						
2.5	1.6	0.30						

Amahusu Shore

Female (11)	2.4	1.7	0.00	Female (2)	2.1	1.1	0.10
	2.7	1.9	0.10		2.3	1.1	0.10
	2.5	1.7	0.10	Male (6)	2.3	1.1	0.30
	2.5	1.7	0.10		2.4	1.2	0.40
	2.7	1.8	0.10		2.1	1.1	0.25
	2.2	1.5	0.10		2.3	1.1	0.25
	2.2	1.8	0.10		2.0	0.9	0.25
	2.0	1.6	0.10		2.5	1.1	0.25
	2.3	1.7	0.05				
	2.3	1.6	0.10				
	2.1	1.5	0.05				
2.4	1.8	0.40					
Male (11)	2.2	1.5	0.45				
	2.5	1.8	0.50				
	2.1	1.5	0.23				
	2.1	1.6	0.25				
	2.3	1.6	0.50				
	2.4	1.6	0.65				
	2.1	1.3	0.40				
	2.2	1.5	0.45				
	2.1	1.4	0.30				
	1.9	1.3	0.25				

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\* Dimensions are in centimeters.

Appendix 14 Distribution of imposex in *Thais luteostoma* in Ambon Bay, 1989

Site <sup>1)</sup>	TS <sup>2)</sup>	MGC <sup>3)</sup>	Number of		RFI (%)	Penis length (mm)		RPS (%)
			female	Total Imposex		Mean	SD	
PFD	L	GS	2	2	100	1.75	0.35	6.40
	P	BTE	10	10	100	2.20	0.92	5.79
GFD	L	GS	1	1	100	1.0		4.21
	P	BTE	8	8	100	1.50	0.53	6.59
GTH	L	GS	1	1	100	3.0		59.27
	P	BTE	6	6	100	3.22	0.58	93.68
AS	L	GS	2	2	100	1.00	0	4.40
	P	GS	11	10	90.9	0.82 (0.90)	0.34 (0.21)	0.87 (1.15) <sup>4)</sup>

Legend:

- 1) PFD = Poka Ferry Dock;  
GFD = Galala Ferry Dock;  
GTH = Galala Tuna Harbour;  
AS = Amahusu Shore.
- 2) TS = Type of specimen;  
L = Live-examined;  
P = Preserved in 4% formaldehyde.
- 3) MGC = Method of gender confirmation;  
GS = Gonad sectioning;  
BTE = Body tissue examination.
- 4) ( ) = Incidence values (excluding 0 values when imposex absent).

## Appendix 15 Results of normality and homoscedasticity tests for female penis length, 1989

### I. Summary statistics of female penis length

Statistic	PFD	Site <sup>1)</sup>		
		GFD	GTH	AS
Sample size (n)	10	8	6	11
Minimum value	0.15	0.10	0.23	0
Maximum value	0.45	0.25	0.40	0.10
Range	0.30	0.15	0.17	0.10
Mean	0.220	0.150	0.322	0.082
Variance ( $\sigma^2$ )	0.008	0.003	0.003	0.001
Standard Deviation	0.092	0.053	0.058	0.034
Coefficient of Skewness (G1)	1.752	0.750	-0.290	-1.544
Coefficient of Kurtosis (G2)	2.042	-0.500	-0.739	1.066
Coefficient of Variation	0.418	0.356	0.182	0.412

<sup>1)</sup> PFD = Poka Ferry Dock; GFD = Galala Ferry Dock;  
GTH = Galala Tuna Harbour; AS = Amahusu Shore.

### II. Lilliefors Test for normality

#### 1. Hypothesis:

$H_0$ : Penis lengths are normally distributed.

#### 2. Level of significant:

$\alpha = 0.05$

#### 3. Criteria:

Reject  $H_0$  if  $P \leq 0.05$

#### 4. Result:

#### 5. Conclusion:

Site	n	$d_{\max}$	P	
PFD	10	0.386	0.000	Reject $H_0$ (non-normal)
GFD	8	0.250	0.160	Accept $H_0$ (normal)
GTH	6	0.189	1.000	Accept $H_0$ (normal)
AS	11	0.432	0.000	Reject $H_0$ (non-normal)

[ $d_{\max}$  = the absolute value of the maximum difference between the relative cumulative frequencies].

### III. Bartlett Test for homoscedasticity

#### 1. Hypothesis:

$$H_0: \sigma^2 \text{ PFD} = \sigma^2 \text{ GFD} = \sigma^2 \text{ GTH} = \sigma^2 \text{ AS}$$

#### 2. Level of significance:

$$\alpha = 0.05$$

#### 3. Criteria:

Reject  $H_0$  if  $P \leq 0.05$

#### 4. Result\* :

$$\text{Approximate } F = 2.883, \quad DF = 3, 1487, \quad P = 0.035$$

[DF = degree of freedom; P = probability]

#### 5. Conclusion:

Reject  $H_0 \Rightarrow$  The variances of penis length of the four sites are heterogeneous.

\* In the case where the cell counts are less than 10, Box's small sample F approximation is used for the probability value rather than Bartlett's usual  $\chi^2$  (Wilkinson, 1988; p. 708).

### IV. Normal Probability Plot

[see page 140; EV = expected value, PL = penis length (cm)]

## Appendix 16

## Results of the gender determination

Specimen number	BTE	GSq	GS	Specimen number	BTE	GSq	GS
GT88#1	M	M	M	PF89#3	M	M	M
GT88#2	F	F	F	PF89#5	F	F	F
GT88#3	F	F	F	PF89#8	M	M	M
GT88#8	M	M	M	GF89#4	M	M	M
GT88#9	F	F	F	GF89#7	M	M	M
GT88#23	F	F	F	GF89#9	F	F	F
GT88#27	M	M	M	A89#1	F	F	F
PF88#2	M	M	M	A89#2	M	M	M
PF88#3	F	?	?	A89#4	F	F	F
PF88#4	F	?	?	A89#5	F	F	F
PF88#12	M	M	M	A89#8	M	M	M
PS88#1	F	F	F	A89#17	F	F	F
PS88#2	M	M	M	A89#28	F	F	F
PS88#3	F	F	F	PF88#5	?	M	?
PS88#11	M	M	M	PF88#6	?	M	?
YS88#2	F	F	F	PF88#8	?	M	?
YS88#9	M	M	M	PF88#10	?	M	?
YS88#12	F	F	F	PF88#14	?	M	?
SR88#2	F	F	F	PF88#15	?	M	?
SR88#9	M	M	M	PF88#16	?	M	?
SR88#24	M	M	M	PF88#17	?	M	?
SR88#30	F	F	F	PF88#19	?	M	?
GA88#3	M	M	M	GA88#6	?	F	F
GA88#4	F	F	F	GF89#8	?	M	M
GA88#7	M	M	M	AS#2	?	M	?
GA88#8	F	F	F	AS#15	?	M	?
GT89#2	M	M	M	AS#16	?	M	?
GT89#5	M	M	M	AS#33	?	M	?
PF89#1	F	F	F	AS#35	?	M	?

Legend:

BTE = Body Tissue Examination (based on secondary sexual characters); GSc = Gonad Squash; GS = Gonad Sectioning; M = male; F = female; ? = undetermined.

GT = Galala Tuna; PF = Poka Ferry; PS = Poka Shore; YS = Yos Soedarso; SR = Slamet Riyadi; GA = Gudang Arang; GF = Galala Ferry; AS = Amahusu Shore.

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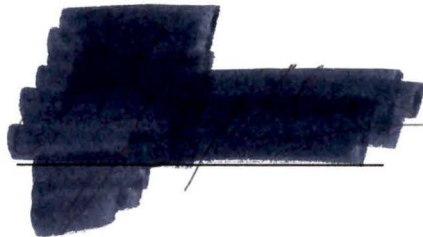
Ellis, D.V. and L.A. Pattisina, 1990. Widespread neogastropod imposex: a biological indicator of global TBT contamination ? Marine Pollution Bulletin **21**: 248-253.

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