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Imposex as an Indication of Butyltin Exposure in the Veined Rapa Whelk (Rapana venosa), a Chesapeake Bay Invader

Ethan Alexander Jestel
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IMPOSEX AS AN INDICATOR OF BUTYLTIN EXPOSURE IN THE
VEINED RAPA WHELK (*RAPANA VENOSA*):
A CHESAPEAKE BAY INVADER

A Thesis
Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

by

Ethan Alexander Jestel
2003
This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Science

Approved, March 2003

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. v
LIST OF TABLES ........................................................................ vi
LIST OF FIGURES ...................................................................... vii
ABSTRACT ................................................................................ viii

INTRODUCTION ......................................................................... 2
  Tributyltin Characteristics ...................................................... 2
  Uses and Sources of Tributyltin ............................................... 4
  Fate of Tributyltin in the Aquatic Environment ....................... 7
  Effects of Tributyltin ............................................................... 9
  Regulations Restricting Tributyltin .......................................... 10
  Imposex Characteristics ....................................................... 10
  Imposex Induction by Tributyltin ........................................... 11
  Mechanism of Imposex Induction ......................................... 12
  The Veined Rapa Whelk (Rapana venosa) ............................ 13
  Veined Rapa Whelk Reproductive Characteristics ................ 15
  Life History of the Veined Rapa Whelk ................................ 17
  Ecological Effects of Veined Rapa Whelks ............................ 17
  Tributyltin, Imposex and Veined Rapa Whelks ..................... 18
  Maternal Transfer of Tributyltin ............................................ 19
  Imposex in the Chesapeake Bay Veined Rapa Whelks ........... 19
  Objectives ............................................................................ 20

MATERIALS AND METHODS .................................................. 22
  Sample Collection ................................................................ 22
  Sample Processing .............................................................. 25
  Egg Collection ..................................................................... 28
  Analytical Method for Muscle Tissue .................................... 28
  Analytical Method for Eggs .................................................. 32
  Method Evaluation ............................................................... 33
  Statistical Analyses ............................................................. 34

RESULTS .................................................................................. 35
  Sample Collection ................................................................ 35
  Sample Selection .................................................................. 36
  Description of Samples ...................................................... 36
  Accumulation of butyltins ................................................... 38
  Seasonal Variability in Butyltin Concentration ..................... 38
  Penis Length and Body Weight Correlation ......................... 43
  Imposex Expression and Butyltin Concentration ................... 48
  Site Comparison .................................................................... 48
  Sex Comparison .................................................................... 57
  Egg Analysis ......................................................................... 57
  Butyltin Concentration and Body Weight Correlation .......... 62
<table>
<thead>
<tr>
<th>DISCUSSION</th>
<th>.................................................................</th>
<th>67</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imposex Expression and Butyltin Concentration</td>
<td>.................................................................</td>
<td>69</td>
</tr>
<tr>
<td>Site Comparison</td>
<td>..................................................................................</td>
<td>73</td>
</tr>
<tr>
<td>Sex Comparison</td>
<td>..................................................................................</td>
<td>75</td>
</tr>
<tr>
<td>Egg Analysis</td>
<td>..................................................................................</td>
<td>76</td>
</tr>
<tr>
<td>Butyltin Concentration and Body Weight Correlation</td>
<td>.................................................................</td>
<td>77</td>
</tr>
<tr>
<td>Monthly Trends in Butyltin Concentration (Females)</td>
<td>.................................................................</td>
<td>80</td>
</tr>
<tr>
<td>Monthly Trends in Butyltin Concentration (Males)</td>
<td>.................................................................</td>
<td>84</td>
</tr>
<tr>
<td>Life History of Female Veined Rapa Whelks (from TBT concentration)</td>
<td>.................................................................</td>
<td>85</td>
</tr>
<tr>
<td>Implications for Biomonitoring</td>
<td>..................................................................................</td>
<td>87</td>
</tr>
<tr>
<td>Future Research</td>
<td>..................................................................................</td>
<td>88</td>
</tr>
<tr>
<td>CONCLUSIONS</td>
<td>..................................................................................</td>
<td>90</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>..................................................................................</td>
<td>91</td>
</tr>
<tr>
<td>VITA</td>
<td>..................................................................................</td>
<td>100</td>
</tr>
</tbody>
</table>
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## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Physical characteristics of sampling regions</td>
<td>23</td>
</tr>
<tr>
<td>2.</td>
<td>Results of statistical tests comparing sites, sexes and egg burdens</td>
<td>37</td>
</tr>
<tr>
<td>3.</td>
<td>Mean TBT and DBT concentrations for veined rapa whelks</td>
<td>39</td>
</tr>
<tr>
<td>4.</td>
<td>Results of statistical tests examining relationships between physical traits and butyltin concentrations</td>
<td>47</td>
</tr>
<tr>
<td>5.</td>
<td>Correlation results between butyltin concentration and body weight</td>
<td>66</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical structures of TBT and derivatives</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Proposed fate of TBT in marine environment</td>
<td>6</td>
</tr>
<tr>
<td>3.</td>
<td>Photograph of veined rapa whelk</td>
<td>14</td>
</tr>
<tr>
<td>4.</td>
<td>Photograph of veined rapa whelk egg mass</td>
<td>14</td>
</tr>
<tr>
<td>5.</td>
<td>Map of sampling region</td>
<td>16</td>
</tr>
<tr>
<td>6a.</td>
<td>External sex verification for imposex females</td>
<td>26</td>
</tr>
<tr>
<td>6b.</td>
<td>External sex verification for males</td>
<td>27</td>
</tr>
<tr>
<td>7.</td>
<td>Method for analysis of muscle tissue for butyltins</td>
<td>29</td>
</tr>
<tr>
<td>8.</td>
<td>Gas chromatogram of TBT standard</td>
<td>31</td>
</tr>
<tr>
<td>9a.</td>
<td>Monthly mean TBT and DBT concentration of JR females</td>
<td>41</td>
</tr>
<tr>
<td>9b.</td>
<td>Monthly mean TBT and DBT concentration of OV females</td>
<td>42</td>
</tr>
<tr>
<td>9c.</td>
<td>Monthly mean TBT and DBT concentration of JR males</td>
<td>44</td>
</tr>
<tr>
<td>9d.</td>
<td>Monthly mean TBT and DBT concentration of OV males</td>
<td>45</td>
</tr>
<tr>
<td>10.</td>
<td>Penis length versus body weight for all samples</td>
<td>46</td>
</tr>
<tr>
<td>11a.</td>
<td>Penis length versus DBT concentration of JR imposex females</td>
<td>49</td>
</tr>
<tr>
<td>11b.</td>
<td>Penis length versus DBT concentration of OV imposex females</td>
<td>50</td>
</tr>
<tr>
<td>11c.</td>
<td>Penis length versus DBT concentration of JR males</td>
<td>51</td>
</tr>
<tr>
<td>11d.</td>
<td>Penis length versus DBT concentration of OV males</td>
<td>52</td>
</tr>
<tr>
<td>12a.</td>
<td>Penis length versus TBT concentration of JR imposex females</td>
<td>53</td>
</tr>
<tr>
<td>12b.</td>
<td>Penis length versus TBT concentration of OV imposex females</td>
<td>54</td>
</tr>
<tr>
<td>12c.</td>
<td>Penis length versus TBT concentration of JR males</td>
<td>55</td>
</tr>
<tr>
<td>12d.</td>
<td>Penis length versus TBT concentration of OV males</td>
<td>56</td>
</tr>
<tr>
<td>13a.</td>
<td>Monthly mean TBT concentrations of JR females and males</td>
<td>58</td>
</tr>
<tr>
<td>13b.</td>
<td>Monthly mean DBT concentrations of JR females and males</td>
<td>59</td>
</tr>
<tr>
<td>14a.</td>
<td>Monthly mean TBT concentrations of OV females and males</td>
<td>60</td>
</tr>
<tr>
<td>14b.</td>
<td>Monthly mean DBT concentrations of OV females and males</td>
<td>61</td>
</tr>
<tr>
<td>15.</td>
<td>Concentration of butyltins in veined rapa whelk egg masses</td>
<td>63</td>
</tr>
<tr>
<td>16.</td>
<td>Mean concentrations of butyltins in JR females throughout the year</td>
<td>64</td>
</tr>
<tr>
<td>17a.</td>
<td>TBT concentrations in water from lower James River (JMS13.1)</td>
<td>83</td>
</tr>
<tr>
<td>17b.</td>
<td>TBT concentrations in water from lower James River (HRH)</td>
<td>83</td>
</tr>
</tbody>
</table>
ABSTRACT

Tributyltin (TBT), an organometallic contaminant toxic to many marine organisms, induces imposex (the imposition of male sexual characteristics on females) in many marine gastropod species. The veined rapa whelk, *Rapana venosa*, is an invasive species in the Chesapeake Bay that has the potential to seriously disturb the local hard clam fishery. Imosex has been observed in Chesapeake Bay veined rapa whelks (Westcott, 2001). The concentrations of TBT and dibutyltin (DBT) in veined rapa whelks were measured to investigate a causal link between imposex occurrence and butyltin exposure.

Veined rapa whelks were collected from the lower James River, Virginia (JR) and from Ocean View, Virginia (OV) in 2002. Individual veined rapa whelks were found to contain parts per billion concentrations of TBT and DBT. Male whelks had higher concentrations of TBT than females. The mean concentration of TBT in female whelks from the JR was lower after egg laying. The presence of TBT and DBT in the eggs indicates the females are able to eliminate butyltins through egg laying.

While TBT has been shown to be the predominant inducer of imposex in marine gastropods, no relationship between TBT concentration and imposex was found for the veined rapa whelks. Significant seasonal variations in TBT concentrations due to changes in feeding rates, metabolism and egg laying make TBT concentration a poor predictor of imposex development in the veined rapa whelk. A significant positive relationship was found between the concentration of DBT in the muscle and the normalized penis length in female and male whelks from JR. DBT may induce imposex in this species or might be a metabolic shadow of historic exposure to TBT. Additional work is needed to evaluate the role of TBT and DBT in imposex development in veined rapa whelks from the Chesapeake Bay.
IMPOSEX AS AN INDICATOR OF BUTYLTIN EXPOSURE IN THE

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INTRODUCTION

Human technology has produced chemical compounds that have never before been substantially involved in the processes of the Earth’s biosphere. Some of these compounds have been manufactured in large quantities. Because it is impossible to know every impact that a particular compound will have in the environment, responsible science must examine the fate and effects of these novel compounds. One compound that has been introduced into the aquatic environment in large quantities is tributyltin (TBT). The extensive production and release of tributyltin into natural systems requires analysis of the possible effects this chemical will have in the environment.

Tributyltin Characteristics

Tributyltin is a general term for a suite of compounds that incorporate the TBT cation (TBT⁺) (Figure 1). Compounds commonly used in industrial applications include TBT chloride (TBTCI), TBT fluoride (TBTF), TBT acetate (TBTOAc) and bis-tributyltin oxide (TBT₂O) (Blunden et al., 1984). Tributyltin is composed of a tin atom covalently bound to three n-butyl chains. The structure of TBT exhibits unusual functionality because the butyl chains impart lipophilic qualities counteracted by the positive charge on the tin atom: TBT has physical and chemical properties of both organic and inorganic compounds. The behavior of TBT in natural systems is difficult to predict because of this organometallic character. One physical property that is used for predicting the uptake rate
Figure 1. Chemical structure of A. TBT, B. degradation products of TBT (DBT and MBT) and C. derivatives of TBT used in analytical method (TPT and TTBT).
A. Tributyltin (TBT)

B1. Dibutyltin (DBT)

B2. Monobutyltin (MBT)

C1. Tripentyltin (TPT)
    Surrogate Standard

C2. Tetrabutyltin (TTBT)
    Internal Standard
of compounds by organisms is the octanol-water partitioning coefficient ($K_{ow}$). The $K_{ow}$ is a property derived by measuring the partitioning of a compound between octanol ($C_{g}H_{ig}O$) and water. Although simplified, this property is meant to reflect the complex process of the migration of molecules from water into biota. Partitioning refers to the movement of molecules between two phases, driven by a change in free energy. Upon reaching steady state, the point at which the rate of movement between phases is the same in both directions, the amount of the compound contained in the octanol is measured and used to calculate the $K_{ow}$. Compounds that are less polar (more organic) will partition to a greater degree into the octanol and consequently have a higher $K_{ow}$. The $K_{ow}$ is often reported as the log $K_{ow}$ because of the potential for a large range of values.

The organometallic nature of TBT causes it to change speciation under varying environmental or experimental conditions. Thus, TBT can have a different $K_{ow}$ under differing pH or salinity conditions. A summary of several experiments testing the partitioning of $\text{TBT}_2\text{O}$, under varied conditions, revealed a range of log $K_{ow}$ from 2.20 to 3.85 (Laughlin, 1996). The reported $K_{ow}$ of TBT indicates that TBT will be moderately available to organisms in the aquatic environment when compared to hydrophobic organic contaminants.

**Uses and Sources of Tributyltin**

TBT is used in a wide range of commercial products, including molluscicides, bactericides, fungicides and wood preservatives (Champ, 1986; Clark et al., 1988). Although, organotin compounds have been known to chemists since the second half of the 19th century, it was not until the 1960’s that TBT was recognized as an excellent
biocide for antifouling paints. Its value was due to its acute toxicity to a wide range of aquatic organisms but low mammalian toxicity (Anderson and Dalley, 1986). This realization prompted large-scale production across the globe and subsequent introduction of large quantities of TBT into the marine environment. The use of TBT in antifouling paints, insecticides, and cleansers creates numerous sources to the aquatic environment, including wastewater treatment facilities and agricultural applications (Bryan and Gibbs, 1991) (Figure 2). The release of TBT from antifouling paints into seawater is the largest environmental concern involving TBT (Fent, 1996).

Antifouling paints are applied to ships to inhibit the growth of fouling organisms. These organisms attach to a vessel’s hull, increasing drag. A 10-micron increase in average hull roughness, caused by fouling organisms, can increase fuel consumption by 0.3 to 1.0 percent (Champ, 1986). A boat with a six month accumulation of fouling organisms can use up to 40% more fuel in order to maintain a normal speed (Hall 1988). In 1975, costs associated with biofouling were estimated to be higher than $1 billion per year (Clark et al., 1988). Early TBT-based antifouling paint formulations were simple mixtures of paint and TBT (Champ, 1986). Upon immersion in seawater, the paints exhibited a high initial release rate of TBT with dramatically reduced biocidal effectiveness over time. Modern self-polishing formulations are characterized by an initial short-lived high release rate followed by a long-term, slow release of TBT. This allows the paint to be effective for extended periods. The recommended application allows for an effective lifetime of up to 5-7 years (Champ, 1986). The longer effective lifetime of TBT-based antifouling paint reduces the frequency that vessels need to be repainted, which can be expensive and costly in labor and loss of useful work time. The
Figure 2. Schematic diagram outlining the proposed fate of TBT in the marine environment.
utility of these TBT-based antifouling paints made them so popular that by 1985 approximately 20-30% of vessels in the world used them (Clark et al., 1988).

**Fate of Tributyltin in the Aquatic Environment**

The most common copolymer used in modern antifouling paints is TBT methacrylate/methylmethacrylate (Anderson and Dalley, 1986; Champ and Seligman, 1996). Upon immersion in seawater, TBT is released from this paint as TBT⁺. The complexity of seawater causes difficulty when determining the exact fate of the TBT⁺ after it is released from the paint. In seawater, TBT might exist in a steady state as equal parts TBT⁺, TBTCI, (TBT)₂CO₃ (tributyltin carbonate), and TBTOH (tributyltin hydroxide) because of the prevalence of ions (Clark et al., 1988). Other researchers have concluded that the slight alkaline nature and composition of seawater will cause the TBT⁺ to form TBTOH, almost exclusively (Blunden et al., 1984; Fent, 1996; Hoch et al., 2003).

Once in the aquatic environment, degradation of TBT is thought to be biologically controlled (Clark et al., 1988; Seligman et al., 1996). Investigations into chemical and photolytic degradation of TBT have determined that in sterile seawater little if any degradation of TBT occurs (Clark et al., 1988; Seligman et al., 1996). Degradation of TBT generally occurs through debutylation to its daughter products: dibutyltin (DBT) and monobutyltin (MBT), and ultimately inorganic tin (Seligman et al., 1996)(Figure 1). Each debutylation results in a less toxic compound, perhaps because of the increasing ionic nature, which would reduce bioavailability (Fent, 1996). Wong et. al. (1982) determined that when tested under identical conditions, the log Kᵦw for DBT was 1.49,
while the log K$_{ow}$ for (TBT)$_2$O was 2.29. Because TBT is more lipophilic than DBT, TBT is more available for uptake by organisms in the aquatic environment.

The relative insolubility of TBT in seawater causes it to partition readily to organic rich particulates, which eventually settle out, creating a large pool of TBT in sediments (Harris, 1996). Research on the sorption properties of TBT has shown that if the concentration of TBT in the water column decreases, it will readily desorb from the sediment (Unger et al., 1988). Dibutyltin also reversibly binds to clay-rich sediments (Hoch et al., 2003). Because the TBT and DBT are reversibly bound to the sediment, the sediment will act as a source of these contaminants if the concentration in the water decreases. Degradation of these sediment pools of TBT is relatively slow. The half-life of TBT in the water column is a few weeks, while the bulk of the TBT, which resides in the sediment, has a half-life on the order of years (Bryan and Gibbs, 1991). Further investigation, by Dowson et al. (1996), revealed that TBT has a half-life of 1-2 years in aerobic sediment and a half-life of decades in anaerobic sediment. After sediment was sterilized with mercuric chloride, there was negligible change in TBT content over an entire year (Dowson et al., 1996). The authors concluded that degradation of TBT in sediment is dominated by microbial activity.

The organometallic nature of TBT (i.e. butyl groups are lipophilic and Sn ion is hydrophilic), results in physical characteristics, which vary greatly depending on the particular environmental conditions of the experiment (Harris, 1996). The half-life of TBT is affected by the pH, salinity, light availability, oxygen content and even the concentration of the TBT in the experimental setup (Fent, 1996; Seligman et al., 1996).
The accumulation of TBT from the environment to an organism is referred to as bioaccumulation (Laughlin, 1996). Because there is no evidence of the TBT cation being actively transported across a membrane, uptake of TBT is driven primarily by the uncharged species (Laughlin, 1996). The uncharged species, probably TBTOH, is taken up by an organism through passive diffusion. This process occurs wherever the organism is in contact with water, sediment or food. Consequently, the overall exchange is dominated by regions of high surface area, such as the gill and the gut.

Effects of Tributyltin

TBT is effective as an antifoulant; however, determination of its value must include assessing its negative effects. In the late 1970’s, TBT was found to leach from antifouling paints into the water column where it affects non-target organisms (organisms which do not grow on ship’s hulls). Concentrations of TBT as low as 300 ng L⁻¹ can cause toxicity, from short-term exposure, resulting in death in many organisms, including copepods, mysids, diatoms and algae (Bushong et al., 1990; Cardwell and Sheldon, 1986; Hall and Bushong, 1996). Toxic effects, from long-term exposure, can occur at 20 ng L⁻¹ TBT and include reduction or inhibition of algal growth, reduction in egg production of copepods, malformed oyster shells and deformation of regenerated claws of fiddler crabs (Bryan and Gibbs, 1991). The marine copepod Acartia tonsa is particularly sensitive to TBT. Survival of Acartia tonsa larvae is reduced at 15-20 ng/L TBT, while developmental effects can occur at 1-2 ng/L (Kusk and Petersen, 1997). Exposure to TBT has also been implicated in the disruption of endocrine processes in gastropods, which can occur at concentrations as low as 1-2 ng L⁻¹ TBT (Bryan and Gibbs, 1991).
Regulations Restricting Tributyltin

Concern for environmental impacts has resulted in government regulation, including bans on the use of TBT in some countries. In 1988, the U.S. restricted the use of TBT antifouling paints on vessels shorter than 25 m, with aluminum hulls exempted (Champ, 2000). The U.S. also regulates the release rate of TBT from paint to be less than 4 μg TBT/cm²/day. The U.S. Environmental Protection Agency has recently proposed the lowering of the regulated marine water quality criteria from 10 ng L⁻¹ to 1 ng L⁻¹ TBT (Brooke et al., 2003). New Zealand and Japan have forbidden all application of TBT containing antifouling paints (Champ, 2000). The International Maritime Organization, a branch of the U.N., has recently issued a global prohibition on TBT for 2008 (IMO, 2001). For many nations, this global regulation will be the first time any restriction has been placed on the use of TBT.

The lower Chesapeake Bay region is a major port of shipping (Seligman et al., 1989). It is also home to the largest coal exporting terminal in the world and the largest naval installation in the western hemisphere (Mann and Harding, 2000). These waters are also heavily used by local recreational and commercial boaters. Before regulations restricting the use of TBT antifouling paint on vessels shorter than 25m, the small local marinas were a significant source of TBT to the marine ecosystem. Large vessels are still a potential source of TBT.

Imposex Characteristics

TBT is suspected to be an endocrine disruptor in gastropods. An endocrine disruptor is a chemical substance that interferes with the production, distribution or
function of hormones (SETAC, 2000). Hormones are important for controlling sleep and wake cycles, modulating growth and controlling reproductive development. In many species of gastropods, including the ones mentioned herein, the sexes are normally separate. In 1970, female dogwhelks (*Nucella lapillus*) were reported with penis like structures (Matthiessen and Gibbs, 1998). A year later, female American mud snails (*Ilyanassa obsoleta*) were identified that had developed a vas deferens (Matthiessen and Gibbs, 1998). This development of male sexual characteristics in females was termed imposex (the imposition of one sex on another), which has since been observed in 118 different species of gastropods (Oberdorster et al., 1998). While penis development is the most noticeable symptom of imposex, advanced stages of imposex can result in the development of a vas deferens, abortion of egg capsules and overgrowth of the genital pore, effectively sterilizing the snail (Folsvik et al., 1999). Blockage of the oviduct by a vas deferens will stop egg laying; however, egg production may continue. The buildup of unlaid eggs may eventually rupture the oviduct wall, possibly killing the snail (Matthiessen and Gibbs, 1998). The combination of imposex induced sterilization and death is thought to be the reason that entire populations of dogwhelks in the United Kingdom were in decline or had completely vanished during the mid-1980s (Matthiessen and Gibbs, 1998).

**Imposex Induction by Tributyltin**

In 1981, it was documented that penis development in females was more frequent in regions close to marinas (Matthiessen and Gibbs, 1998). This evidence, coupled with experimental results, led to a link between TBT exposure and imposex in gastropods. Shortly after regulatory action was enacted to limit the release of TBT into the marine...
environment, many populations of dogwhelks, that were previously endangered, recovered (Matthiessen and Gibbs, 1998). Before restrictions limiting the use of TBT in Canada, 8 of 11 sites that were examined contained 100% imposex affected females (Reitsema et al., 2002). A decade later only one of those 8 sites still had 100% imposex affected females (Reitsema et al., 2002).

Although, there is little doubt that the dominant environmental cause of imposex in most gastropods is TBT (Reitsema et al., 2002), other factors have been found that cause imposex. Under laboratory conditions, environmental stress, acetic acid, copper and nonylphenol have all been shown to cause imposex, although not always in a clear and consistent manner (Nias et al., 1993; Reitsema et al., 2002). Triphenyltin (TPhT), which is also used in antifouling paints, has also been linked to imposex. Direct injection of TPhT was shown to induce imposex in rock shell (Thais clavigera), a gastropod (Horiguchi et al., 1994), and field data indicated that TPhT has some effect on imposex in the same species (Shim et al., 2000). However, laboratory exposure and injection of TPhT did not induce imposex in dogwhelks (Bryan et al., 1988).

**Mechanism of Imposex Induction**

The exact mode of action of TBT on the endocrine system of organisms is not yet fully understood. Numerous experiments have been conducted investigating the link between TBT and imposex. Direct injection of TBT into dogwhelks initiated penis growth in females (Spooner et al., 1991). One study determined that mud snails that were classified as imposex had a greater retention of unmetabolized testosterone, a male hormone, than non-imposex snails (Oberdorster et al., 1998). The authors indicated that
periwinkles (*Littorina littorea*), which do not exhibit imposex, have a much higher excretion rate of testosterone metabolites. If elevated testosterone is causing imposex then it is possibly the result of competitive inhibition of aromatase by TBT (Matthiessen and Gibbs, 1998). Aromatase converts testosterone to 17β-estradiol, a female hormone. Another theory is that the retention of testosterone and its metabolites might not be the cause of imposex, but rather a result of it. Changes in testosterone metabolism are not apparent until later stages of imposex, after penis and vas deferens development (Oberdorster and McClellan-Green, 2000). Oberdorster and McClellan-Green (2002) suggest that TBT may be initiating the release of a penis morphogenic factor (PMF), a neurohormone, in the female gastropods. The authors theorize that perhaps TBT is causing imposex through a combination of neurohormone induction and aromatase inhibition. Tributyltin may initiate the production of the PMF beginning the development of a penis (Oberdorster and McClellan-Green, 2002). In order to continue the development of the penis and other secondary sexual characteristics, TBT may inhibit aromatase, causing a buildup of testosterone in female gastropods.

**The Veined Rapa Whelk (Rapana venosa)**

The veined rapa whelk is a large, predatory gastropod that was first observed in the Chesapeake Bay in the summer of 1998 (Harding and Mann, 1999)(Figure 3). It is native to the Sea of Japan, the Yellow Sea, the East China Sea and the Gulf of Bohai. The rapa whelk has been introduced into the Black Sea, the Adriatic Sea, Aegean Sea, has recently been observed in Uruguay and is now growing in numbers in the Chesapeake Bay (Harding and Mann, 1999; Savini et al., 2002). These snails were probably introduced to the Chesapeake Bay by the ballast water of either commercial or military
Figures 3 and 4: Adult veined rapa whelk shell (length = 165 mm) and rapa whelk egg mass. Copyrights for photographs, respectively, are:

2001, Juliana Harding, Virginia Institute of Marine Science and

Figure 3.

Figure 4.
ships. Research continues to determine the exact numbers and geographic range of this organism (Molluscan ecology program website, 2002). Whelks have been collected from fishermen through a bounty program established by the VIMS molluscan ecology group. To date, over 5200 specimens and many egg capsules have been collected (Figure 4). Many of these whelks have come from the lower James River region (Figure 5). Most of these specimens have been larger than 70 mm in shell length. One possible reason for this is that fishermen working soft substrate for shellfish are not catching small rapa whelks because juvenile whelks settle onto hard substrate (Harding and Mann, 2001). The large numbers of snails and viable egg capsules collected from the lower Chesapeake Bay, indicate that this population is capable of reproduction. Research is underway to determine whether the population is large enough to be self-sustaining (Harding and Mann, 1999; Mann and Harding, 2000).

**Veined Rapa Whelk Reproductive Characteristics**

Veined rapa whelks normally have two distinct sexes and there is no evidence that they change sex at any point in their lifetime (Westcott, 2001). Rapa whelks larger than 78 mm are able to reproduce (Westcott, 2001). Mating can occur from February through June (Westcott, 2001). Rapa whelks form mating piles in captivity, where females probably mate with several different males. The male will insert his penis into the mantle cavity of the female and deposit sperm, which is then stored for future egg fertilization (Westcott, 2001). Egg laying in rapa whelks occurs from mid-May to mid-August (Westcott, 2001). Captive whelks lay an average of 10 egg masses per female (Ware, 2002) (Figure 4). On average, egg masses are made up of 150 egg cases that are bound
Figure 5. Map of Hampton Roads, VA, U.S.A. with sampling sites indicated (JR-James River and OV-Ocean View). Key sites of potential TBT contamination are marked. Bridges: JRB-James River bridge, MMBT-Monitor Merrimac bridge tunnel, HRBT-Hampton Roads bridge tunnel and CBBT-Chesapeake Bay bridge tunnel.
together at their bases and anchored to hard substrate (Ware, 2002). On average, each egg case from an adult whelk contains 1440 eggs (Ware, 2002).

**Life History of the Veined Rapa Whelk**

Veined rapa whelks hatch from eggs as planktonic larvae (Chung et al, 1993)(Mann and Harding, 2000). Once settled on a hard substrate (i.e. a piling or oyster reef) the larvae probably feed mostly on oyster spat (*Crassostrea virginica*) (Harding, 2002). During their development, the young whelks can make periodic trips to the sediment in order to feed or attempt burrowing (Harding, 2002). Once large enough to eat hard clams, adult rapa whelks probably use soft sediment habitat exclusively. The females must return annually to some form of hard substrate because eggs are laid on hard substrate. They are nocturnal hunters and probably spend the rest of the time burrowed in sediment where they are able to both move and mate (Harding and Mann, 1999). As winter approaches, rapa whelks probably migrate to deeper sediment or deeper water (Wu, 1988).

**Ecological Effects of Veined Rapa Whelks**

Veined rapa whelks have been maintained in captivity for extended periods. They have been observed to preferentially consume hard clams but will also consume oysters. In captivity, adult whelks consume one large clam every two weeks, on average (Harding et. al, 2003). There is great potential for rapa whelks to disrupt the ecologically and economically important clam fishery in the lower Chesapeake Bay (Harding and Mann, 1999; Savini et al., 2002). Furthermore, the native oyster population is already severely impaired and restoration efforts could be compromised if predation pressures increased.
Even in death, veined rapa whelks have an ecological effect. Their shells are both thicker and boxier than the native whelks. Hermit crabs that reside in rapa whelk shells are better protected from predation and are able grow to a larger size than was previously possible (Harding and Mann, 1999).

**Tributyltin, Imposex and Veined Rapa Whelks**

Veined rapa whelks might be exposed to TBT through several pathways, including exchange from the water or sediment across the gills or by exposure in the gut from contaminated food or ingested sediment. Oyster borers (*Lepsiella scobina*) from New Zealand, were found to have similar concentrations of TBT as the oysters upon which they were feeding (King et al., 1989). The authors suggest that; based on this finding, direct exchange from the water was the primary route of TBT exposure for this species. A study by Stickle et. al. (1990) found that file dogwinkles (*Nucella lima*) bioaccumulated TBT from both food and water in a direct relationship with the concentration and duration of exposure. This leaves the primary route of exposure of TBT to veined rapa whelks in question because there are multiple possibilities. Adult whelks consume hard clams when available. Because hard clams are poor degraders of TBT and have a high bioconcentration factor (amount of TBT in dry tissue divided by the amount in the water) (Bryan and Gibbs, 1991), veined rapa whelks will be exposed to TBT through consumption of clams. Monthly monitoring of waters in the Hampton Roads region indicates average concentrations of 1-2 ng L$^{-1}$ TBT$^+$ (Unger, 2002). Two sediment samples taken in the James River had concentrations of 9 and 36 μg/kg TBT$^+$ (dry weight) (Espourteille, 1988). Veined rapa whelks from this region will be
chronically exposed to TBT through both the water and the sediment in which they live, eat, and breed.

The extent of bioaccumulation of butyltins by rapa whelks from the Chesapeake Bay is currently unknown. For exposure to result in bioaccumulation, the rate that the whelks are able to clear their bodies of the contaminant (elimination) must be slower than the influx of new contaminant (uptake). Periwinkles, another gastropod species, had a bioconcentration factor for TBT of over 14,000 (Bryan and Gibbs, 1991). Possible mechanisms for the elimination of TBT include degradation to debutylated species (DBT, MBT and inorganic tin) and maternal transfer from females to their eggs. The ability of the veined rapa whelk to metabolize TBT is currently unknown. Although gastropods lack sophisticated metabolic pathways for the efficient breakdown of contaminants (Bryan and Gibbs, 1991), two gastropod species, the dogwhelk and the file dogwinkle, metabolize TBT to DBT and MBT (Lee, 1996).

Maternal Transfer of Tributyltin

Tributyltin was found to undergo maternal transfer from Japanese medaka (Oryzias latipes), a fish, to their eggs (Nirmala et al., 1999). In addition, only TBT was transferred in significant amounts: DBT and MBT, although present in the female, were not transferred to the eggs in detectable concentrations.

Imposex in the Chesapeake Bay Veined Rapa Whelks

Imposex in the veined rapa whelk has been previously documented in the Chesapeake Bay population by E. Westcott (Westcott, 2001). This study of gonadal histology concluded that there were no apparent reproductive differences between female
whelks and imposex female whelks, despite the growth of a penis. This conclusion was reinforced by findings that eggs laid by normal females and imposex affected females did not significantly differ in morphology, hatch success, or number of eggs laid (Ware, 2002). While these results indicate that imposex females can successfully mate and lay eggs under the current environmental conditions, the long-term viability of offspring of imposex affected whelks has not been investigated.

Objectives

Human activities during the past several decades have introduced large quantities of TBT into the marine environment. This compound is acutely toxic to a broad range of marine organisms at low concentrations and has many chronic effects. Imoex induction in gastropods has been linked to TBT exposure. The veined rapa whelk is not native to the Chesapeake Bay. Its impacts on the Bay, as well as, the Bay’s impacts on the whelk are not yet known. It is certain that the rapa whelk is exposed to TBT, but what has not been shown is the biological effect, if any, TBT exposure is having on the veined rapa whelks.

The first objective of this study was to determine if rapa whelks accumulate butyltins in their tissues. If whelks are exposed to butyltins, but do not accumulate them, then attempts to link tissue concentration and imposex induction will be fruitless.

The second objective was to evaluate whether butyltins are inducing imposex in rapa whelks. In particular, this study focused on linking the degree of imposex in this species with the tissue concentration of TBT and DBT. Furthermore, the concentration of butyltins in the tissues of males was examined. The development of masculine traits
resulting from exposure to butyltins in female gastropods is well documented; however, the effect of exposure to butyltins on males has not been researched as extensively. This study also contrasted the occurrence of imposex in whelks collected from two spatially distinct regions of the lower Chesapeake Bay.

The third objective was to compare concentrations of butyltins in male and female rapa whelks. Differences in concentrations of butyltins may be attributed to maternal transfer of butyltins into eggs. Analysis of eggs for TBT and DBT might indicate whether female rapa whelks are eliminating butyltins through egg laying.

The final objective was to examine how concentrations of butyltins changed as whelks age. The balance between uptake and elimination of TBT and DBT will determine how concentrations of these contaminants change within the whelks as they grow. Differences in the relative concentrations of TBT and DBT within the tissues of whelks of different ages (different weights) may indicate whether uptake or elimination processes are dominant.
MATERIALS AND METHODS

Sample Collection

Veined rapa whelks were collected from the lower Chesapeake Bay through a bounty program established by the VIMS Molluscan Ecology group (Harding and Mann, 1999). Whelks were collected from commercial patent tong clammers, crab dredgers and crab pot fishermen working in the lower Chesapeake Bay. Samples for this study were collected between December 2001 and May 2002 from two different areas: the James River region (JR), located between the Monitor-Merrimac Bridge Tunnel and the James River Bridge, and the Ocean View region (OV), located off Willoughby Spit between the Hampton Roads Bridge Tunnel and the Chesapeake Bay Bridge Tunnel (Figure 5). The JR is characterized by a proximity to heavy industry; especially shipyards that handle TBT paint. The OV is less industrialized and more susceptible to tidal flushing from the lower Bay that will likely lead to lower concentrations of TBT. These two areas form the east and west boundaries of the region in which the majority of whelks have been collected. The two regions differ in several physical features (Table 1).

The number of samples that were collected was limited by the catch rate of the fishermen and the availability of the resources necessary to collect them from the fishermen. Initially, a target quota was set at about 60 individuals of each sex class, from both sites. Of these 60 whelks, 30 whelks that covered a wide range of body weights were selected.
Table 1. Physical characteristics of James River and Ocean View regions

(from Ware, 2002). Used with permission.
<table>
<thead>
<tr>
<th>Region</th>
<th>Salinity (ppt)</th>
<th>Depth (feet)</th>
<th>Sediment Type</th>
</tr>
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<tr>
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<td>20-60</td>
<td>Soft Sand</td>
</tr>
<tr>
<td>Ocean View</td>
<td>25-35</td>
<td>18-30</td>
<td>Hard Sand</td>
</tr>
</tbody>
</table>
for analysis. Collection of specific sexes was hampered by the need to kill the whelk before sexing was possible. Upon arrival at VIMS, the whelks were frozen at just under 0°C. Snails were frozen for no more than one month before processing. Periodically the frozen whelks were processed to obtain physical data and biological samples. Shell length and total whelk weight were recorded before the whelk was removed from the shell. Once the shell was removed, the body (all soft tissue), viscera and muscle weights along with penis length and the appearance of the gonad were recorded. The foot (muscle tissue) of each whelk was individually frozen. Samples were frozen for a maximum of 7 months before analysis.

The foot tissue was selected for analysis for a number of reasons. Whelks were sampled from December 2001 through May 2002, during which time the whelks were preparing for or were actively mating. Metabolism, diet and reproductive development may cause fluctuations in the distribution and magnitude of the concentration of butyltins among the various tissues of the whelk. The size and composition of the muscle should change less than other tissues, particularly the gonad, as a function of breeding status. Research by Power and Keegan (2001) has shown that in the red whelk (*Neptunea antiqua*) the foot tissue contained the third lowest concentration of TBT among six different body tissues. Information on how pools of TBT among the different body tissues change with time is not available. The foot, being a large portion of the body (from 10 to 50% of total body weight), makes it easily identified and excised from surrounding tissue during dissection. Finally, since each analysis requires several grams of tissue, the foot muscle provides enough analytical material for the tissue concentration of TBT and DBT of individual whelks to be measured.
Sample Processing

External sexing of animals was performed by Dr. J. Harding (VIMS Molluscan Ecology). This classification was made based on experience and the size of the penis relative to the body weight of the animal (Harding, 2002). A small animal with a relatively large penis was deemed a male while a similarly sized animal with a relatively small penis was an imposex female. Normal females were aphallic. Because there was a continuum of penis lengths and body weights, some animals possessed penises of intermediate size for their body weights. Whether these were small males or large imposex females was unknown. External examination of the gonad was not definitive. The sex of a whelk can be determined through histological analysis of the gonad; however, the cost of performing histological analyses on the whelks in this project was deemed prohibitive. During a previous experiment, Westcott performed histological examination of 96 veined rapa whelks from the lower James River region in order to validate the external sex determination (Westcott, 2001). External sexing was found to be accurate in every whelk except for one. The data was used as a benchmark to sex rapa whelks for this study. The penis lengths and body weights from Westcott’s study were plotted and 99% prediction lines were established (Figures 6a and 6b). The physical measurements for the whelks from this study were then overlaid on this data. The prediction lines had to be extrapolated somewhat to account for animals that fell outside the size range of the whelks from Westcott’s study. Whelks that either fell above the upper prediction line of the imposex females or below the lower prediction line of the males were not used. The penis length of each whelk was used as a metric for the degree
Figure 6a. External sex verification for imposex females. Rapa whelks from current study are plotted against rapa whelks of known sex from Westcott (2001) in order to verify external sexing. Circled whelks were not analyzed.
Penis length (mm) vs. Body weight (g)

- Black dots: Imposed Females (Westcott, 2001) (n=30)
- White circles: Imposed Females (this study) (n=100)

99% Prediction Limit
Figure 6b. External sex verification for males. Rapa whelks from current study are plotted against rapa whelks of known sex from Westcott (2001) in order to verify external sexing. Circled whelks were not analyzed.
of imposex. Penis length has been shown to be a useful absolute indicator of imposex (Folsvik et al., 1999; Oberdorster et al., 1998), as well as being a good relative indicator of varying degrees of imposex (Bryan et al., 1989).

**Egg Collection**

Female rapa whelks that were collected from the JR were held in Taylor floats in the York River from May to August 2001 (Ware, 2002). These whelks were monitored twice a week and once an egg mass was laid it was collected. Half of each egg mass was used for fecundity and morphology analysis while the other half was frozen. At the conclusion of the experiment, the whelks were sacrificed, sexed and the muscle tissue was frozen. Egg masses and muscle tissues were analyzed from four females, three of which exhibited imposex. At most, three egg masses were analyzed from each whelk: the first, second and final egg mass that was laid during the summer.

**Analytical Method for Muscle Tissue**

The analytical method for rapa whelk foot tissue is a modification of the technique for sediment analysis for TBT in use by Dr. M. Unger’s lab at VIMS (Unger, 1996). The method begins with an acidic digestion followed by an extraction using organic solvents. The extracted butyltins are quantified with gas chromatography. This procedure is summarized below and illustrated in Figure 7.

Foot tissue was thawed, rinsed with deionized water and homogenized. Five grams were then weighed into a 50 mL Teflon® centrifuge tube with a 1-2 gram sample set aside for determination of the sample’s dry weight. A tripentyltin chloride (TPTCl)
Figure 7. Summary of analytical method for measuring butyltins in rapa whelk tissue.
Homogenized tissue (5g)

Add TPTCl and HCl
Sonicate for 30 minutes

Add 5mL Toluene and 5mL Hexane

Shake for 1 hour
Centrifuge for 20 minutes

Discard tissue residue

Derivatize and clean up

Supernatant
Add 4BT
GC-FPD

Repeat once with fresh solvent
solution in hexane was added to each 5 gram sample. Five mL of concentrated HCl was added to each tube and the samples were sonicated for 30 minutes with venting and shaking. This partially liquefied the samples to facilitate extraction of the analytes during shaking. Five mL of a 0.1% tropolone solution in toluene and 5 mL of hexane were then added to each sample. The samples were shaken on an automatic wrist shaker for 1 hour and centrifuged for 20 minutes at 7500 rpm. Once the supernatant solvents were pipetted into a glass centrifuge tube, toluene and hexane were added again and the samples were shaken and centrifuged a second time. The supernatants were then combined and the tissue residue was discarded. The tripentyltin (TPT), TBT, DBT and MBT ions were derivatized by the addition of a Grignard reagent, hexylmagnesium bromide. The Grignard reagent was then neutralized with acid and the aqueous layer was separated from the solvent and discarded. The extract was passed through a column of Florisil® and sodium sulfate and then concentrated to 10 mL. The internal standard, tetrabutyltin (TTBT), was then added to the samples that were further reduced to 0.1 mL.

The samples were analyzed with a Varian® 3300 gas chromatograph equipped with a dual-flame flame photometric detector (FPD) with a hydrogen rich flame. The GC was setup according to Dr. M. Unger’s lab (Unger, 1996). The tin compounds are converted into tin-hydrides within the flame of the detector and then measured photometrically based on the emission of tin, in the order that they eluted. The tetra-alkyl nature of these derivatized compounds not only provided stability, but also imparted excellent separation based on the molecular weights of the respective derivatives. The TTBT eluted first, while the trihexylbutyltin (MBT derivative) was the last to come off the column (Figure 8).
Figure 8. Gas chromatogram of TBT standard used for calibration and monitoring of the gas chromatograph. Peaks are labeled with respective compound names before derivitization.
Retention time (minutes)
Since the FPD converted all of the tin containing analytes into tin-hydride, the retention time of the analytes was the only characteristic that uniquely identified each analyte. The retention times for each compound were determined by analyzing standards. All analyte signals were quantified using the magnitude of the TPT surrogate standard signal. The similar structure between the TPT and the target analyte, TBT, should have caused the TPT to be lost in the same proportion as the TBT throughout the analytical procedure (Figure 1). Thus, by spiking a known concentration of TPT onto the tissue before extraction, the relative amounts of TPT and TBT at the beginning of the analytical procedure should be unchanged at the conclusion of the procedure. By establishing a ratio between the final FPD signal of the TPT and its known initial concentration, the original concentration of TBT in the tissue was calculated. The addition of a known concentration of TTBT immediately before injection on the gas chromatograph, allowed for the calculation of the percent recovery of the TPT. Any difference between the calculated TTBT concentration, based on the signal of TPT, and the known spiked concentration was directly attributable to a loss of TPT during the analysis.

Analytical Method for Eggs

Veined rapa whelk eggs were analyzed using the same method as the muscle with a few modifications. Egg masses were thawed, rinsed with deionized water and then divided into clumps of about 10 egg cases. These clumps were then mixed by hand and randomly selected and placed in a 50 ml Teflon® centrifuge tube until the required sample weight was reached. When available, 3 grams of eggs were analyzed. A few samples were limited so that only 2 grams were analyzed. Experiments indicated that analytical results from 2 and 3 gram samples were similar. The tripentyltin chloride was
spiked onto the egg cases followed by 20 mL of concentrated HCl. The samples were
sonicated for 30 minutes, manually shaken and stored in the refrigerator overnight. Tubes
were then shaken vigorously to break up the softened egg cases, releasing the eggs from
the cases. The samples were then extracted and analyzed using the tissue method
previously described.

Method Evaluation

Quality control data reveal how accurate and dependable a method is for analysis.
Typical recoveries of TPT, the surrogate standard, ranged from 60 to 90%. The percent
recovery is a measure of how much analyte is lost throughout the method. The method
was linear ($R^2$ for TBT=0.99 and DBT=0.95) for spiked samples up to 300 ng g$^{-1}$ (dry
weight) TBT or DBT, which encompassed all but a small number of highly contaminated
samples. Six replicate samples of a muscle tissue were analyzed and the mean percent
deviation, a measure of precision, was 2% for TBT and 18% for DBT. The method
detection limit for TBT was 2.9 ng g$^{-1}$ (dry weight) and for DBT was 3.5 ng g$^{-1}$ (dry
weight). These data indicate that measurements using this method were reliable.

Comparison with other methods was difficult because of the scarcity of full
reports on the quality control of published methods. When compared to the available data
for published methods, the current method was similar for DBT analysis and slightly
more precise for TBT analysis (Bryan et al., 1986; Mensink et al., 1997; Stickle et al.,
1990).

The method for egg analysis proved reliable, although it was not tested as
thoroughly as the muscle method. Recoveries of internal standard were in the same range
as the tissue method. Three replicate samples, from a single egg mass, had less than 1% mean deviation for TBT and 9% mean deviation for DBT.

Statistical Analyses

Physical measurements of whelks of different sexes and from different collection regions were compared using a two-way analysis of variance (ANOVA), with an $\alpha$ set at 0.05 a priori. Main effects included body weight, penis length, the concentration of TBT ([TBT]) and the concentration of DBT ([DBT]). Analysis of covariance (ANCOVA), with $\alpha=0.05$, was used to analyze the relationship between the concentration of butyltins and penis length for groups that exhibited significant seasonal effects. A simple linear regression was used if the analysis was not affected by seasonal effects. The Tukey multiple comparison test was used following ANOVA for all post-hoc comparisons. Correlations between the concentration of butyltins and the body weight of the whelks were measured with a Pearson product moment correlation coefficient. The ratio of [DBT]/[TBT] was transformed with a logarithm to satisfy the assumption of normality. No other data was transformed because it met basic assumptions of normality and homogeneity of variance. The SAS System®, version 8.00, was used to conduct statistical analyses.
RESULTS

Sample Collection

A total of 512 veined rapa whelks were collected from fishermen working in areas of interest within the lower Chesapeake Bay during this study. From JR, 104 males, 63 imposex females, and 15 normal females were collected. From OV, 237 males, 35 imposex females, and 58 normal females were collected. Commercial fishermen working the areas of interest collected the whelks as bycatch and provided them to VIMS along with collection information, in exchange for a bounty. For this project, whelks were collected from December 2001 through May 2002. State regulations that govern commercial shellfisheries determined when and where sampling occurred. Whelks were collected from the JR by fishermen using patent tongs (January through March) and crab pots (April and May). Whelks were collected from OV by fishermen using crab dredges (February and March) and crab pots (April and May).

Most of the sex classes were collected at a steady rate during the sampling period; however, OV whelks were collected predominately during March, April, and May. No males that were collected during May were kept for this study because a sufficient number of males had been collected by then.

There was a large difference in the proportion of imposex affected females to normal females in the samples collected from the two regions. Of the females collected
from JR, 81% were imposex females, while only 38% of OV females were imposex females. If TBT was the dominant environmental cause of imposex in the veined rapa whelk, the preponderance of imposex females in the JR may indicate greater exposure to TBT. The samples were collected as bycatch from fishermen, which is not a random collection strategy, so the sample might not accurately represent the whole whelk population.

**Sample Selection**

A limited number of samples could be analyzed due to time and resource limitation. Samples for analysis were selected to cover a wide range of body weights and penis lengths. Whelks with body weights less than about 30 g were not analyzed because they provided insufficient muscle tissue for accurate analysis. Whelks that fell outside of the 99% prediction limits for sex determination were also rejected for analysis (Figures 6a and 6b). The seasonal variability, which will be examined later, was not recognized until late in the analysis, so only a few whelks were selected for analysis based on the month that they were collected.

**Description of Samples**

The smallest snail analyzed was a JR imposex female that had a shell length of 88 mm and a 3.5 mm penis. The largest snail was an OV imposex female that was 171 mm in length with a particularly small penis length of 3.7 mm. All of the whelks analyzed were probably reproductively active because they were all larger than 78 mm in length (Ware, 2002; Westcott, 2001). Whelks of the same sex, from the two regions, did not have significantly different body weights (Table 2). Males from the JR were significantly
Table 2. Results of statistical tests comparing physical traits between sites and sexes, comparing [butyltin] between males and females within sites, comparing the [TBT] to [DBT] in rapa whelk eggs and comparing the [TBT] in females before and after egg laying (ANOVA, $\alpha=0.05$). Significant results are marked with an asterisk. Abbreviations: BW-body weight, PL-penis length, M-male, IF-imposex females, F-both normal and imposex females.
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<th>df</th>
<th>p-value</th>
<th>Conclusion (if null hypoth. is rejected)</th>
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<td>[TBT]_m &gt; [TBT]_f</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[DBT]</td>
<td>1</td>
<td>0.03*</td>
<td>[DBT]_m &gt; [DBT]_f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JRM vs JRF(Mar)</td>
<td>TBT</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>[TBT]_m &gt; [TBT]_f</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[DBT]</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>[DBT]_m &gt; [DBT]_f</td>
</tr>
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<td></td>
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<td>JRM vs JRF(Apr)</td>
<td>TBT</td>
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<td>0.74</td>
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</tr>
<tr>
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<td>[DBT]</td>
<td>1</td>
<td>0.79</td>
<td>Null hypothesis</td>
</tr>
<tr>
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<td>OVM vs JRF(Mar)</td>
<td>TBT</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>[TBT]_m &gt; [TBT]_f</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[DBT]</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>[DBT]_m &gt; [DBT]_f</td>
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<tr>
<td></td>
<td></td>
<td>OVM vs JRF(Apr)</td>
<td>TBT</td>
<td>1</td>
<td>0.02*</td>
<td>[TBT]_m &gt; [TBT]_f</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>[DBT]</td>
<td>1</td>
<td>0.03*</td>
<td>[DBT]_m &gt; [DBT]_f</td>
</tr>
<tr>
<td>[TBT] and [DBT] in eggs are same</td>
<td>ANOVA</td>
<td>Eggs</td>
<td>[TBT] vs [DBT]</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>[TBT]<em>(eggs) &gt; [DBT]</em>(eggs)</td>
</tr>
<tr>
<td>Pre and post egg laying have same [TBT]</td>
<td>ANOVA</td>
<td>JRF(May vs Aug)</td>
<td>TBT</td>
<td>1</td>
<td>0.03*</td>
<td>[TBT]<em>(May) &gt; [TBT]</em>(Aug)</td>
</tr>
</tbody>
</table>
larger than the females, while males and females from the OV region did not have significantly different body weights (Table 2). Throughout this study, females are used as an inclusive term for the combination of imposex and normal females. As would be expected, males from both regions had significantly longer penis lengths than imposex females (Table 2). Imposex females from the JR had significantly longer penis lengths than those from the OV site, although the penis lengths of the males from the two sites were not significantly different (Table 2).

**Accumulation of Butyltins**

All of the rapa whelk muscle tissue that was analyzed contained detectable concentrations of TBT (Table 3); however, two of the samples contained DBT that was below the detection limit, both of which were OV normal females. Thus, rapa whelks did accumulate both TBT and DBT in their muscle tissue. Unless otherwise stated, all tissue concentrations are expressed as ng g$^{-1}$ TBT$^+$ or DBT$^{++}$ dry weight (DW). No overall comparisons between the concentration of butyltins in different sexes or sites will be presented here because a strong monthly variability was evident. Overall comparisons would be skewed by sampling because the whelks were not collected evenly across the sampling months.

**Seasonal Variability in Butyltin Concentration**

Water temperatures started at 5°C for both regions and increased to 18°C at OV and 21°C at JR from January through May 2002 (CBP, 2002). As water temperatures rise the activity level and feeding rate of whelks held in captivity have been observed to increase (Harding, 2002).
Table 3. Mean TBT and DBT burdens for analyzed rapa whelks from the JR and OV collection sites within the lower Chesapeake Bay (n=sample size).
<table>
<thead>
<tr>
<th></th>
<th>TBT (ng g(^{-1}) DW)</th>
<th>DBT (ng g(^{-1}) DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (std. dev.)</td>
<td>Mean (std. dev.)</td>
</tr>
<tr>
<td>JR imposex females</td>
<td>42 (59)</td>
<td>27 (17)</td>
</tr>
<tr>
<td>(n=34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JR true females</td>
<td>55 (88)</td>
<td>28 (27)</td>
</tr>
<tr>
<td>(n=14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JR males</td>
<td>43 (15)</td>
<td>43 (19)</td>
</tr>
<tr>
<td>(n=29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OV imposex females</td>
<td>42 (41)</td>
<td>43 (53)</td>
</tr>
<tr>
<td>(n=34)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OV true females</td>
<td>22 (29)</td>
<td>16 (14)</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OV males</td>
<td>60 (101)</td>
<td>28 (24)</td>
</tr>
<tr>
<td>(n=30)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mean TBT and DBT concentrations in the JR females showed monthly fluctuation (Figure 9a). From January through March, TBT concentrations remained relatively unchanged and were generally low with a small variance. A large change occurred between March and April when the mean TBT concentration increased more than five fold. This increase was accompanied by a large increase in variance. This trend continued into May when the mean TBT concentration increased again, but to a lesser degree with no change in the variance of the sample. The TBT concentration during May was higher than the March concentration, with April being intermediate and similar to both. The mean DBT concentration of whelks from JR did not show as much seasonal change. The mean DBT concentration from January through May gradually increased each month, but the months were not significantly different. The only notable change occurred between March and April when the variance of the sample increased. Much like the TBT, there was no change in the variance of mean DBT concentrations from April to May in the JR females.

Female whelks from OV exhibited a similar trend to the JR females in monthly mean TBT concentrations, although with lower magnitudes (Figure 9b). The TBT concentrations in the OV females were relatively low and did not fluctuate from February through March. The TBT concentrations were approximately 30% lower than the JR females. From March to April, the TBT concentration doubled and was accompanied by a large increase in variance. The concentration of TBT in the muscle of the OV females almost quadrupled from April to May.

Unlike the JR whelks which exhibited a gradual increase in [DBT] over the 5 months sampled, the [DBT] in the OV whelks were steady at around 15 ppb during
Figure 9a. Monthly mean TBT and DBT concentrations in JR females (both normal and imposex). Means denoted with different letters are statistically different (Tukey’s multiple comparison test, $\alpha=0.05$). Surface water temperatures, from the Chesapeake Bay Program’s water quality web site, for the lower James River (2002) are also reported. Error bars are ± standard deviation.
Figure 9b. Monthly mean TBT and DBT concentrations in OV females (both normal and imposex). Means denoted with different letters are statistically different (Tukey’s multiple comparison test, α=0.05). Surface water temperatures, from the Chesapeake Bay Program’s water quality web site, for OV (2002) are also reported. Error bars are ± standard deviation.
The graphs show the mean concentration of TBT (left) and DBT (right) over different collection months. The x-axis represents the collection months: January (n=6), February (n=10), March (n=25), April (n=16), and May (n=25). The y-axis represents the mean concentration (mg g⁻¹ DW) for TBT and DBT. The concentration of TBT and DBT increases significantly from January to May. Water temperature is also shown, with a peak in May. The bars indicate statistical significance, with letters A and B suggesting different groups based on statistical analysis.
February, March, and April (Figure 9b). From April to May, the DBT concentration changed dramatically with an almost six fold increase and a large increase in variance. Only a single female was sampled in December from OV and it showed elevated concentrations of both TBT and DBT. This was the only snail analyzed from December and no corroborating evidence is available to validate these high concentrations.

The tissue concentrations of both TBT and DBT in the JR males showed very little seasonal variation (Figure 9c). TBT concentrations remained unchanged from January to April. DBT concentrations were consistent except for a moderate increase in March, followed by a decrease in April. Only the March and April DBT concentrations were significantly different from each other.

The OV males that were analyzed were all collected during February, March, and April (Figure 9d). Much like the JR imposex females, there was an almost four fold increase in the TBT concentration from March through April and a similar increase in the variance. The DBT concentration decreased slightly from March to April. Mean concentrations for both TBT and DBT were not significantly different for either March or April.

**Penis Length and Body Weight Correlation**

There was a significant positive correlation between the penis lengths and the body weights for males and imposex females from both sites (Figure 10)(Table 4). Penis lengths were normalized to body weight because samples covered a wide range of body weights. Body weight is a volumetric measurement and will increase as the cube of the
Figure 9c. Monthly mean TBT and DBT concentrations in JR males. Means denoted with different letters are statistically different (Tukey’s multiple comparison test, α=0.05). Surface water temperatures, from the Chesapeake Bay Program’s water quality web site, for the lower James River (2002) are also reported. Error bars are ± standard deviation.
Figure 9d. Monthly mean TBT and DBT concentrations in OV males. Means denoted with different letters are statistically different (Tukey’s multiple comparison test, α=0.05). Surface water temperatures, from the Chesapeake Bay Program’s water quality web site, for OV (2002) are also reported. Error bars are ± standard deviation.
Figure 10. Penis length related to body weight for males and imposex females from the OV and JR regions.
Table 4. Results of statistical tests determining if the penis lengths and body weights of the rapa whelks are correlated (Pearson’s method, $\alpha=0.05$) and determining if the normalized penis length is related to the [TBT] or [DBT] of the rapa whelks (linear regression with ANOVA or ANCOVA, $\alpha=0.05$). Significant results are marked with an asterisk. Abbreviations: BW-body weight, PL-penis length, M-male, IF-imposex females, colmonth-collection month.
<table>
<thead>
<tr>
<th>Null hypothesis</th>
<th>Test</th>
<th>Group</th>
<th>Factors</th>
<th>df</th>
<th>p-value</th>
<th>Conclusion, if null is rejected</th>
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<td>PL and BW are not correlated</td>
<td>Pearson product moment</td>
<td>JRIF</td>
<td>PL vs BW</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>PL and BW are correlated</td>
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<tr>
<td></td>
<td>correlation coefficient</td>
<td>OVIF</td>
<td>PL vs BW</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>PL and BW are correlated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JRM</td>
<td>PL vs BW</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>PL and BW are correlated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OVM</td>
<td>PL vs BW</td>
<td>1</td>
<td>&lt;0.01*</td>
<td>PL and BW are correlated</td>
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<td></td>
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<tr>
<td>Norm. PL not related to [butyltins]</td>
<td>Linear Reg. with ANOVA</td>
<td>JRM</td>
<td>[DBT]</td>
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<td>0.04*</td>
<td>Positive relationship: norm. PL and [DBT]</td>
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<td>[TBT]</td>
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<td>JRM</td>
<td>[DBT]</td>
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<td>&lt;0.01*</td>
<td>Positive relationship: norm. PL and [DBT]</td>
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<td></td>
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<td>[DBT]*Colmonth</td>
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<td>Colmonth is significant factor</td>
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<td></td>
<td></td>
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<td>[TBT]*Colmonth</td>
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<td>Relationship is different btwn. Colmonths</td>
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<tr>
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<td>0.51</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03*</td>
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<td></td>
<td></td>
<td></td>
<td>[TBT]*Colmonth</td>
<td>1</td>
<td>0.97</td>
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<tr>
<td></td>
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<td>OVIF</td>
<td>[DBT]</td>
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<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>[DBT]*Colmonth</td>
<td>1</td>
<td>0.34</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>[TBT]</td>
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<td>0.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05*</td>
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<td>[TBT]*Colmonth</td>
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<td>0.08</td>
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<td></td>
<td></td>
<td></td>
<td>[DBT]*Colmonth</td>
<td>3</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[TBT]*Colmonth</td>
<td>3</td>
<td>0.44</td>
<td></td>
</tr>
</tbody>
</table>
linear penis length, so the normalized penis length metric was set as

\[ \text{penis length(body weight)}^{1/3}. \]

**Imposex Expression and Butyltin Concentration**

The [DBT] in the muscle tissues of the JR imposex females showed a significant relationship with the normalized penis length (Figure 11a)(Table 4). The collection month had a significant effect on the concentration of DBT for the JR imposex females (Table 4). This was true for all of the analyzed groups, except the JR males.

There was a significant positive relationship between normalized penis lengths and [DBT] in the JR males (Figure 11c)(Table 4). A simple linear regression was used to test this relationship because an ANCOVA indicated that the collection month did not have a significant effect on the relationship.

Neither the OV males nor imposex females indicated a significant relationship between normalized penis lengths and [DBT] (Figures 11b and 11d)(Table 4). There were not any significant relationships between the [TBT] in the muscle and the normalized penis lengths for any of the whelk groups (Figures 12a-d)(Table 4).

**Site Comparison**

Of the whelks analyzed, 71% of OV females and only 38% of JR females were collected during April and May. Overall, comparisons of the concentration of butyltins in whelks from different regions might be biased because samples were not available or selected for analysis uniformly among the different months.
Figure 11a. Normalized penis length related to the concentration of DBT in JR imposex females, according to collection month during 2002.
Figure 11b. Normalized penis length related to the concentration of DBT in OV imposex females, according to collection month during 2001-2. Imsect females were not available from OV during January.
Figure 11c. Normalized penis length related to the concentration of DBT in JR males, according to collection month during 2002. Males were not sampled during May because sufficient males had already been collected.
Figure 11d. Normalized penis length related to the concentration of DBT in OV males, according to collection month during 2002. Males were not available from OV during January. Males were not sampled during May because sufficient males had already been collected.
Figure 12a. Normalized penis length related to the concentration of TBT in JR imposex females from Chesapeake Bay, according to collection month.
Figure 12b. Normalized penis length related to the concentration of TBT in OV imposex females from Chesapeake Bay, according to collection month.
Figure 12c. Normalized penis length related to the concentration of TBT in JR males from Chesapeake Bay, according to collection month.
Figure 12d. Normalized penis length related to the concentration of TBT in OV males from Chesapeake Bay, according to collection month.
Similar to the imposex females, differences in sampling time might be the reason that the JR males showed a significant relationship between normalized penis length and [DBT] while the OV males did not. Of the whelks analyzed, 67% of the OV males and only 10% of the JR males were collected during April.

**Sex Comparison**

Only whelks collected in the same month were compared because the collection month of the whelks had a significant effect on the concentration of butyltins in most cases. A monthly comparison of the concentration of butyltins of males and females revealed some differences. Males from the JR usually had higher muscle concentrations of both TBT and DBT than JR females (Figures 13a and 13b)(Table 2). During February and March, the amount of TBT in the JR males was almost three times as high as what the females contained. TBT concentrations did not differ in JR males and females collected during January, but DBT concentrations in the males were twice as high as the females. During April, there was no difference in the concentrations of either TBT or DBT in the tissues of males and females from JR. During March and April, the OV males had higher concentrations of both TBT and DBT, than the OV females. (Figures 14a and 14b)(Table 2).

**Egg Analysis**

A key difference between the life histories of male and female rapa whelks is egg laying. Egg laying may be a major route of elimination of lipophilic compounds, like
Figure 13a. JR female and male monthly mean TBT concentrations. Significant
differences between sexes during a particular month are indicated with an
asterisk. Error bars are ± standard deviation.
Figure 13b. JR female and male monthly mean DBT concentrations. Significant differences between sexes during a particular month are indicated with an asterisk. Error bars are ± standard deviation.
Figure 14a. OV female and male monthly mean TBT concentrations. Significant differences between sexes during a particular month are indicated with an asterisk. Error bars are ± standard deviation.
Figure 14b. OV female and male monthly mean DBT concentrations. Significant differences between sexes during a particular month are indicated with an asterisk. Error bars are ± standard deviation.
TBT and DBT, for female rapa whelks. During most months that whelks were collected, female rapa whelks had less TBT and DBT in their tissues than males (Table 2). This disparity may be a result of females reducing their body burden through egg laying during the summer.

As described in the methods, eggs laid by field caught rapa whelks were analyzed to determine the concentration of butyltins. All analyzed eggs had detectable concentrations of both TBT and DBT (Figure 15). Egg masses laid at the beginning of the summer had higher concentrations of butyltins than those laid at the end of the summer. In addition, the [TBT] was significantly higher than the [DBT] for all eggs (Table 2). The whelks that laid these eggs were processed at the conclusion of the summer and their muscle tissues were analyzed for TBT and DBT. The mean [TBT] of female whelks that were caught from the JR in May 2002, probably prior to any egg laying, was significantly higher than the mean [TBT] of analyzed whelks from the conclusion of the egg laying experiment in August 2001 (Table 2)(Figure 16). The concentration of TBT in whelks at the beginning of the egg laying season was an order of magnitude greater than the concentration of TBT in the whelks at the end of the egg laying season. There was no change in the mean [DBT] between the whelks before and after egg laying. Between August and January, the mean TBT concentration more than tripled, while the DBT concentration decreased by a little over 30 percent (Figure 16).

**Butyltin Concentration and Body Weight Correlation**

To determine if there was any trend between the concentration of butyltins in the muscle of the whelks and the age of the whelks, [TBT] and [DBT] were analyzed for
Figure 15. The burden of butyltins in the first, second and final egg masses laid during the summer of 2001 by four JR female rapa whelks. Egg laying occurs from mid-May through early August. Each of the four whelks laid between 9 and 18 egg masses.
Figure 16. Mean [butyltins] in JR female rapa whelks collected during May (before most egg laying), August (after egg laying), and January (after overwintering).
Collection month

Mean [butylin][ng g⁻¹ butylin DW)

May 2002  
(n=10)

August 2001  
(n=4)

January 2002  
(n=7)

TBT+

DBT++
correlation with the body weight of the whelks. The seasonal variability in the concentrations of the butyltins required that each month be analyzed separately for the various whelk groups and the different butyltins.

Correlations between the body weight of the whelks and the concentration of TBT ([TBT]) and DBT ([DBT]) varied depending on the collection month. The only consistent trends were that body weight and [DBT] were significantly correlated during March for all of the groups and that body weight and [TBT] were not correlated in any of the groups (Table 5). The body weights of JR males were significantly correlated with both DBT concentration and the log of the ratio between [DBT] and [TBT] (Table 5). The concentration of butyltins in males from the JR did not show a butyltin concentration dependence on collection month, so they were not analyzed on a monthly basis.
Table 5. Results of statistical test determining if [TBT], [DBT] or the log of the ratio between [DBT] and [TBT] are correlated with the body weights of the rapa whelks (Pearson’s method, α=0.05). First value is the correlation coefficient and the second is the p-value. Significant results are marked with an asterisk. Abbreviations: M-male, F-both normal and imposex females.
<table>
<thead>
<tr>
<th>Group</th>
<th>Month</th>
<th>N</th>
<th>TBT</th>
<th>DBT</th>
<th>Log ([DBT]/[TBT])</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRM</td>
<td>Jan-Apr</td>
<td>29</td>
<td>0.07/0.73</td>
<td>0.71/0.001*</td>
<td>0.63/0.01*</td>
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<tr>
<td>JRF</td>
<td>January</td>
<td>7</td>
<td>0.30/0.51</td>
<td>0.79/0.03*</td>
<td>0.01/0.98</td>
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<tr>
<td></td>
<td>February</td>
<td>12</td>
<td>0.19/0.56</td>
<td>0.49/0.10</td>
<td>0.23/0.48</td>
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<tr>
<td></td>
<td>March</td>
<td>11</td>
<td>-0.53/0.09</td>
<td>0.73/0.01*</td>
<td>0.86/0.01*</td>
</tr>
<tr>
<td></td>
<td>April</td>
<td>8</td>
<td>-0.06/0.89</td>
<td>-0.05/0.90</td>
<td>0.04/0.91</td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>10</td>
<td>-0.38/0.28</td>
<td>-0.29/0.42</td>
<td>0.32/0.38</td>
</tr>
<tr>
<td>OVM</td>
<td>March</td>
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<td>-0.74/0.02*</td>
<td>-0.22/0.57</td>
</tr>
<tr>
<td></td>
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<td>0.16/0.49</td>
<td>0.37/0.11</td>
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<tr>
<td>OVF</td>
<td>February</td>
<td>6</td>
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<td>0.65/0.16</td>
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<tr>
<td></td>
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<td>0.68/0.03*</td>
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DISCUSSION

Rapa whelks’ exposure to butyltins resulted in accumulation of TBT and DBT in the muscle tissue (Table 3). Statistical analysis revealed that the collection month of the whelk had a significant effect on the butyltin tissue concentrations for all of the sample groups, except the JR males. Because samples were not collected uniformly during the sampling period, statistical comparisons could only be made for whelks collected during the same month.

A general comparison can be made between tissue concentrations of TBT and DBT in rapa whelks and published results from other imposex affected gastropods. Field studies examining imposex occurrence in other gastropods found that imposex in mud snails occurred at whole body tissue concentrations as low as 20 ng g\(^{-1}\) TBT, while dogwhelks were affected by imposex at tissue concentrations that were below 100 ng g\(^{-1}\) TBT (Bryan and Gibbs, 1991). About half of the imposex female rapa whelks from the current project contained [TBT] that was less than 20 ng g\(^{-1}\), some as low as 3 ng g\(^{-1}\). These data suggest that imposex induction in rapa whelks occurs at lower concentrations than in either mud snails or dogwhelks.

Comparisons of concentration data from this project and others must be tempered with the knowledge that data from this project dealt only with the muscle tissue while others used whole body homogenates. Although the muscle tissue has been shown to
have a relatively low concentration of TBT when compared to other body tissues (Power and Keegan, 2001b), the muscle often makes up almost half of the body weight of a whelk. The muscle can contain the largest amount of TBT (Bryan et al., 1993; Power and Keegan, 2001b). Comparisons of muscle concentrations to whole body concentrations might be justified if the concentration in the muscle can be shown to strongly reflect the concentration in the whole body.

Differences in life histories of gastropod species might complicate direct comparisons. A survey that collected red whelks on a monthly basis, determined that there were no obvious seasonal trends in the concentrations of TBT or DBT in the tissues (Power and Keegan, 2001b). This is in contrast with the seasonal trend presented here for rapa whelks. The red whelk fishery, in the Irish Sea, catches whelks throughout the year using baited pots (Power and Keegan, 2001a). This implies that, unlike the rapa whelk, the red whelk does not undergo an overwintering period of low activity. Therefore, a direct comparison of red whelk and rapa whelk tissue concentration of TBT must be done with an understanding of seasonal TBT concentration fluctuations.

The strong monthly variability, that is evident in the rapa whelk data, was surprising in its magnitude. Based on data from Westcott (2001), all of the whelks analyzed for this project were of reproductive age and the gonadal status of the whelks should be either developing or mature. For males, these stages involve the development of sperm producing tissues and active sperm. For females, eggs are either being produced or are fully developed and are ready for fertilization and laying. Egg laying begins around mid-May. As whelks prepare for breeding, the size and composition of their tissues can change. This alteration in tissue structure should facilitate a shift in storage of lipophilic
contaminants. The gonads in particular must develop new reproductive tissues, which are not present during the rest of the year. This new tissue is not in steady state with the rest of the body and contaminants will partition between the new and old tissues to reestablish steady state. The concentrations of the old tissues will decrease, as the new tissues accumulate butyltins. The shift in tissue concentrations that might be facilitated by preparations for breeding was one of the motivations for analyzing the muscle tissue. The large size of the muscle tissue should reduce the effect that reproductive development has on the butyltin concentration in the muscle tissue. In addition, to minimize seasonal effects on tissue concentrations, samples were collected as rapidly as possible beginning in late December 2001. Collection was continued until sufficient samples for this project were acquired, which ended up requiring five months. Ideally, the samples would have been collected in a shorter period; however, the wide distribution, thin coverage, and reliance on commercial fishermen for sampling of the whelks delayed their collection. The sample collection rate was governed by the number of fishermen participating in the project and state regulations that mandate which regions were open to certain methods of fishing at certain times of the year.

**Imposex Expression and Butyltin Concentration**

The significant positive relationship between normalized penis length and the concentration of DBT in the muscle tissue of the JR imposex females can be interpreted in several ways. The first is that the DBT was inducing imposex in the rapa whelks. The fact that the concentration of DBT could be used to roughly predict the normalized penis length, suggests that DBT was causing the penis development. One way to test this relationship would be through laboratory exposure experiments and even direct injection
of DBT into whelks, to see if imposex occurs. Although laboratory exposures have not been conducted on rapa whelks, research on imposex induction in dogwhelks included exposure to DBT. Seawater exposure and injection of DBT to dogwhelks did not induce imposex (Bryan et al., 1988). Although there is the possibility that DBT may induce imposex in rapa whelks and not in dogwhelks, this information raises doubts about the role of DBT in inducing imposex in rapa whelks.

Another possible explanation for the relation between DBT and the normalized penis length is that the TBT induced the imposex and the concentration of DBT was merely a metric of past TBT exposure. Exposure to TBT is believed to be the primary environmental cause of imposex in gastropods (Reitsema et al., 2002). Analysis of tissue concentrations of butyltins in rapa whelks is essentially a snapshot of a long-term process. At the time of analysis, data on the degree of imposex and TBT and DBT concentrations are collected. The amount of time that is necessary for a certain amount of TBT to induce a particular degree of imposex is unknown. What can be surmised is that the degree of imposex at the time of analysis is not a result of just the tissue concentration of butyltins at that moment in time, but a result of a long-term exposure to TBT over the course of many years (Stickle et al., 1990). Rapa whelks from the Chesapeake Bay are believed to live more than 8 years (Harding, 2002). The tissue concentration of butyltins in the whelks will change depending on uptake and elimination processes. The current research has already shown that females eliminate TBT through maternal transfer to their eggs. Thus, the TBT concentration at the time of imposex induction has probably changed by the time analysis occurred. The result of this is that the degree of imposex at the time of analysis is more likely a result of TBT exposure in the past than the TBT
concentration at the time of analysis. There is some evidence that development of imposex is a slow process that does not peak for many months after the initial TBT exposure, under experimental conditions. File dogwinkles that were exposed to high concentrations of TBT, showed intensification of imposex symptoms over a 10 month period during which they were held in seawater with low-concentration TBT contamination (Stickle et al., 1990). Control females that were held in the same seawater with low-concentration TBT contamination did not exhibit any significant imposex development. The authors conclude that the dogwinkles must require several years to recover from TBT exposure. Thus, imposex expression at the time of analysis may be the result of TBT exposure as much as 10 months before analysis. This is the argument for why the tissue concentration of TBT at the time of analysis is not related to the degree of imposex, but it leaves the question of why the DBT concentration is significantly related to the degree of imposex.

As previously stated, the degree of imposex at the time of sampling might be the result of past TBT exposure. A laboratory exposure study by Bryan et al. (1988) with dogwhelks determined that twice as much DBT was generated from internal degradation of TBT, than by direct uptake from solution. If this trend is conserved under field conditions and for the rapa whelk, the majority of DBT in a rapa whelk may have been directly derived from previously bioaccumulated TBT. As a result, the concentration of DBT at the time of analysis may act as a metabolic shadow of the amount of TBT the whelk has been exposed to over the course of its lifetime.

The potential exists that TBT exposure only induces imposex in juveniles, which is then perpetuated through their adulthood. A study on imposex induction in common
whelks (*Buccinum undatum*) collected from a pristine area revealed that only juveniles exposed to TBT developed imposex, while adults were unaffected (Mensink et al., 1996). If TBT only induces imposex in juvenile rapa whelks, than the imposex expression present in the adult rapa whelks is completely due to historic TBT exposure. This previously accumulated TBT may then have been metabolized to produce the concentration of DBT that is present in the whelk at the time of analysis.

The relationship between the normalized penis length and the [DBT] in the JR imposex females is important as an indicator of a possible relationship between the exposure of butyltins and the induction of imposex. Although this relationship does not establish a cause-effect link between TBT exposure and imposex development, it does provide some evidence that such a link does exist for this species. Caution must be used when trying to establish a cause-effect relationship. There are numerous endocrine disrupting contaminants in the marine environment that may be affecting rapa whelks. Combinations of effects from several different contaminants may act to induce imposex, possibly in conjunction with other environmental stressors. The determination of the relationship between imposex development and these other contaminants and stressors will further clarify the role that TBT plays in imposex development.

Males from JR showed a significant relationship between the [DBT] and the normalized penis length. Results from other species are mixed. Male mud snails were found to have significantly greater penis expression as tissue TBT concentrations increased (Bryan et al., 1989). Contrary to what the current project has found in the JR males, the penis lengths of both male dogwinkles and male dogwhelks were unaffected by exposure to TBT (Bryan et al., 1988; Bryan et al., 1989).
The ability of females to undergo masculinization may indicate that the hormonal reproductive systems of the two sexes are similar. Tributyltin may induce imposex by initiating the release of a penis morphogenic factor (PMF), followed by a competitive inhibition of aromatase, which converts testosterone to estradiol (Oberdorster and McClellan-Green, 2002). If the endocrine system of the rapa whelk is similar to other investigated gastropods, some general observations can be made. Normally female rapa whelks are aphallic and should have an active aromatase system. The initiation of the PMF and inhibition of aromatase would be a severe alteration to their endocrine system. In male rapa whelks, the PMF should be naturally occurring and the action of aromatase is probably already limited. If TBT acts as described, the initiation of PMF release and the inhibition of aromatase would further enhance testosterone concentrations and perhaps penis length. The contradiction between the effect seen in rapa whelks and other species can be explained by the complexities of endocrine modulation and the potential for significant differences between life histories of different species.

Site Comparison

The JR imposex females exhibited a significant relationship between normalized penis length and DBT concentration, but the OV imposex females did not. The JR and OV regions, while spatially separated, are not distant enough to expect the two groups to be biologically distinct. Physical characteristics of the two regions differ in a number of ways (Table 1). These differences could be sufficient to alter some aspect of the whelk’s local ecology and cause the disparity in relationships.
Closer examination of these data suggests that the reason is probably at least in part due to sampling differences between the two sites. More OV imposex females, than JR imposex females, were collected during May, a period of elevated and variable tissue concentrations (Figures 11a-b) and increasing water temperatures. In the OV imposex females, all of the whelks collected in March and April have less than 35 ng g\(^{-1}\) DBT, but during May, only a few of the whelks have concentrations that are below 35 ng g\(^{-1}\) DBT. The DBT concentration of the OV imposex females was particularly high during May. The normalized penis lengths of the OV imposex females are significantly related to the concentration of DBT in their muscle tissue during March (ANOVA, df=5, F=9.1, p=0.04). Perhaps if a greater percentage of OV imposex females had been collected during February and March, the relationship would have been significant in both the JR and OV imposex females. The only way to fairly compare these two regions would be to collect whelks at the same rate during sampling because of the dependence of the relationship between normalized penis length and DBT concentration on collection month. The reliance on commercial fishermen for sampling collection for the current research made it impossible to predetermine sampling rate.

There was a significant relationship between normalized penis length and DBT tissue concentration in the males collected from JR, but not in the males from OV (Figures 11c-d). In addition, the DBT tissue concentrations in the JR males were not significantly affected by collection month, but the OV males were. The explanation for this difference could stem from differences in sampling. Much like the imposex females, a larger percentage of males were collected during April from OV than from JR. Collection of the OV males over a longer period might have yielded more information.
Sex Comparison

The tissue concentration of TBT and DBT in the males was higher than the females from both regions during most of the months that were examined (Figures 13a-b and 14a-b). One possible explanation of this is that the males are eating more than the females and are consequently exposed to higher concentrations of butyltins. The exact feeding behavior of the males and females in the field is unknown; however, it is unlikely that the males and females feed at different rates. Both the males and the females will likely eat as much and as fast as possible because of the advantages (including protection from predation and improved success in mating and feeding) that size confers upon the whelks. The difference in the TBT concentration between the male and females is probably a result of differences in elimination processes, rather than uptake rates.

Something must occur between May and February that allows the females to reduce their TBT concentration because the females have relatively low concentrations of TBT in February and the female’s concentrations increase until May. A study of the tissue concentration of TBT in the red whelk revealed that TBT concentrations in females experienced a sudden decrease between March and May, which coincides with the period of egg laying for the red whelk (Power and Keegan, 2001b). Egg laying in the rapa whelks may explain the decrease in TBT concentration that occurs between May and February. Females produce large quantities of eggs which are very high in lipid, a possible sink for TBT in the body, and which are eliminated from the body when they are laid. This may be a significant route of elimination of lipophilic contaminants.
Egg Analysis

Rapa whelk egg masses were analyzed to determine their content of TBT and DBT (Figure 15). Since these eggs are produced in the female, the female’s body concentration should be the source of any contamination. Egg masses were collected from several JR females from May through August 2001. The analyzed egg masses revealed that both TBT and DBT are eliminated by the female whelks in their egg masses. The eggs contained significantly more TBT than DBT. This was expected since TBT is more lipophillic than DBT, which should cause the TBT to preferentially partition to the lipid rich eggs. Once the egg mass is laid, the concentration of butyltins in the egg mass is eliminated from the whelk’s body and thus the overall tissue concentration is diminished. As the next egg mass is readied for laying, the amount of TBT and DBT in the whelk is lower than was available for the previous egg mass. Thus, the first egg mass that a rapa whelk lays should have more TBT and DBT than the next and so on. Since, rapa whelks are able to lay numerous egg masses during the course of a summer, the amount of TBT and DBT in the first egg mass should be markedly different from the amount in the final egg mass. Concentrations of TBT in eggs did generally decrease through the course of the summer and the first and last egg masses had different concentrations of TBT and, to a lesser degree, DBT.

The high concentration of TBT in the egg masses should result in decreased tissue concentrations of the females. Unfortunately, tissue analysis can only be performed after death, so the individual change in tissue concentration before and after egg laying cannot be directly determined. Comparisons can be made between the muscle concentration of the 2001 JR females after a summer of egg laying and the 2002 JR females, before egg
laying. This comparison reveals that there is a significant difference between the female TBT concentration before and after egg laying (Figure 16)(Table 2). DBT concentrations did not change between these two groups, presumably because uptake and elimination mechanisms balanced.

A simple mass balance of the amount of TBT lost from the female whelks between May and August and the amount of TBT that was measured in the egg masses laid by individual snails revealed that these two quantities are of the same order of magnitude. The amount lost by the snails was approximately 10 μg TBT and the amount in the eggs was approximately 30 μg TBT. While this mass balance relied on several assumptions, including a constant decrease in TBT concentration of the egg masses that were not analyzed, the fact that these two values were of the same order of magnitude suggests that a significant portion of the TBT that was lost by the female rapa whelks over the summer can be attributed to maternal transfer of TBT into their eggs.

TBT concentration in the muscle tissues of JR females more than tripled from August to January (Figure 16). During this time, the whelks are still active and feeding, but are not laying eggs. The continued uptake of TBT from the environment may surpass elimination of TBT, which results in an increase in tissue concentration.

Butyltin Concentration and Body Weight Correlation

As whelks age, their body weights will increase. Although whelks with the same body weight are not necessarily the same age, body weights can give a first approximation of the relative ages of whelks. Over time, the relative rates of uptake and elimination will determine the body concentration of butyltins. If uptake rates are
dominant than accumulation will occur. Should this trend persist over the many years of a whelk’s life, the amount of butyltins accumulated each year will be compounded and larger (older) whelks will contain more butyltins than smaller (younger) whelks.

The concentration of DBT in both the JR and OV females were significantly correlated with the body weight of the whelks during March (Table 5). The reason that this correlation exists only in March is unknown. Perhaps the life history of the whelks is such that the rates of uptake and accumulation balance during most of the year except during specific months. More information is required about the various rates and mechanisms governing uptake and elimination.

The DBT concentration of the JR males showed a significant correlation with body weight (Table 5). This indicates that the analyzed JR males retained similar amounts of DBT as they aged. This is probably a result of similar diet and feeding rates. However, the OV males did not show any significant correlation between body weight and DBT concentration (Table 5). Sampling may be a cause of this difference. JR males were collected relatively evenly across four months, while the majority of the OV males were collected in April.

The lack of correlation between the body weight and TBT for any of the whelks may be the result of TBT’s high variability between months, which may obscure any relationship (Table 5).

Correlations of log ([DBT]/[TBT]) with the body weight of the whelks on a monthly basis did not provide any clear information. The females showed significant correlations between the log ([DBT]/[TBT]) and the body weight during different months
(Table 5). This could be a result of environmental factors, but the reason is unknown. The possibility also exists that egg laying occurs during different months for the females from the JR and OV, which may cause the difference in correlation. The previously presented monthly variability in the TBT concentration may hide any correlation during other months. Furthermore, the OV males did not show any correlation between the log of the ratio of DBT to TBT, while the JR males did show a significant overall correlation (Table 5).

The significant correlation in the JR males may provide some insight into the balance of uptake and elimination processes in the males. The positive correlation suggests that as the JR males age, their tissue concentration has a greater proportion of DBT than TBT. Since males are unable to eliminate butyltins through egg laying, elimination rates are probably governed by metabolic processes. For this ratio to be correlated with body weight, every year that a JR male lives, there must be a greater net amount of DBT retained than there is TBT. The larger increase in TBT than DBT that occurs during April and May in the females may indicate that increased activity from warmer waters increases the uptake of TBT, more than DBT. Assuming uptake rates are similar in the males and females, the males will actually accumulate more TBT than DBT. The only way greater exposure of TBT could lead to greater accumulation of DBT in males is if the overall elimination rate (including metabolism) of TBT is faster than the overall elimination rate of DBT.
Monthly Trends in Butyltin Concentration (Females)

Examination of the trends in the monthly means of the different sex and regional classes reveals some hints about the life history of the rapa whelk. The monthly mean TBT tissue concentrations of the females from both sites do not change significantly from February through March (Figures 9a-b). The whelks were experiencing cold-water temperatures during that time and may be primarily inactive, with feeding slowed or halted and respiration at a minimum. As a result, TBT uptake should be low and governed primarily by direct uptake from the water across the gills and skin.

From March to April, the mean body concentration of TBT in females increased (Figure 9a-b). Whelks collected during April experienced warmer water temperatures, which would cause increased activity and food consumption, resulting in an increase in TBT exposure. As water temperatures rise during the Spring, whelks in captivity do become progressively more active and food consumption increases rapidly (Harding, 2002). The variance of the female mean TBT concentrations was high during April and May. During April, there are a small percentage of snails that have elevated TBT concentration. Since the percentage of snails that have elevated concentrations increases during May, these snails have probably just been active for a longer time and their elevated concentrations are reflecting increased exposure from water and food. The minimum concentration has also increased from April to May, which may indicate that more of the whelks have become active.

The dramatic increase in TBT concentration and variance from March to April may provide information about the route of uptake for the TBT. In captivity, the whelks
are almost immobile with very little feeding during the winter (usually November through March) (Harding, 2002). Respiration, metabolism, and feeding rates decrease during this period. From January to March, the TBT concentrations of the JR imposex females do not change significantly. The TBT concentration increases dramatically during the Spring, perhaps to reflect an increase in whelk activity in response to increasing water temperatures. While increased respiration would cause the whelk to be exposed to more dissolved TBT, the size of the increase in TBT concentration suggests a bigger change than the increase in respiration would produce. The increased water temperatures also cause the whelks to begin feeding as they recover from a long winter of low food consumption. Furthermore, the whelks are preparing for egg laying, which is an energy intensive process. This suggests that between March and April the food consumption of the whelks not only returns to a rate to sustain growth, but also probably is accelerated as the whelks stockpile energy for egg laying. This may be evidence that the large increase in TBT concentration is predominately the result of consumption of TBT contaminated food. Food consumption might also be accompanied by inadvertent ingestion of sediment. Further information on the feeding rates and routes of exposure of TBT is required.

The monthly variability in butyltin body concentrations might be the result of seasonal shifts in environmental concentrations of TBT and DBT, rather than the life history of the whelks. The warming waters, which may signal an increase in whelk activity also triggers boat owners to prepare for the summer. Some of this preparation involves painting boats with new antifouling paint. While the use of TBT containing paint is regulated, application on aluminum-hulled boats is still permitted and illegal use
surely persists. Furthermore, release rates of TBT from paints increase in warmer temperatures (Seligman et al., 1996), suggesting that the input of TBT from boats would increase as waters warm. Water monitoring data from the James River does not support this theory. TBT concentrations in the water in the James River did not change very much from March to May 2001 at two stations in the lower James River region (Unger, 2002)(Figures 17a-b).

The only significant difference between the monthly mean TBT concentrations in the females collected from the JR and OV site is the magnitude of the concentrations. During April, the JR females had a higher mean TBT tissue concentration than the OV females. Since body weights of the collected whelks were not significantly different between the two sites, the higher concentration in the JR females may be a result of greater TBT and DBT exposure. Unfortunately, no environmental TBT concentrations are available for the OV region to test this theory. The close proximity of the JR region to a large shipyard and a major coal terminal may result in greater TBT exposure for whelks collected from the JR region, compared to whelks from OV. Since the OV site is closer to the main stem of the Chesapeake Bay, this region may experience a higher rate of flushing which may reduce TBT exposure through dilution with water that is lower in TBT. Assuming TBT is the dominant environmental inducer of imposex, the lower percentage of imposex-affected females collected from the OV region indicates that there is more bioavailable TBT in the JR region.

Both OV and JR females had high mean concentrations of TBT and DBT in May and low concentrations in January or February. They either are able to metabolize TBT faster than it is taken in or there is some other mechanism for elimination of TBT from
Figures 17a and 17b. TBT concentrations for surface water from two stations (JMS13.1 and HRH) in the lower James River during 2001.

(From Unger, 2002, with permission).
Figure 17a.

Figure 17b.
their muscle tissue; potentially egg laying. Furthermore, the mean TBT concentrations in
the spring are significantly higher than those during the winter, while DBT
concentrations do not change significantly. This may be a direct result of the whelks
eliminating more TBT through egg laying than DBT, during May through August.

DBT concentrations continue to rise from March until May. This may be a result
of continued metabolism of TBT in addition to direct uptake of DBT from the
environment.

The mean DBT concentration in May, of the OV females, was high. This may be
a result of increased uptake of DBT from some unknown source. If it were from
metabolic degradation of TBT, the DBT concentrations in the JR females would probably
have been higher, since their TBT concentrations were higher than the OV females.

Monthly Trends in Butyltin Concentration (Males)

If warmer waters cause increased feeding and respiration that results in an
increased tissue concentration of TBT in the females, than a similar result would be
expected for the males. There is no significant change in the monthly mean TBT
concentration in JR males from January to April (Figures 9c-d). While the mean TBT
concentration in April has not increased significantly, the slight increase in the mean TBT
concentration may preclude a larger increase from April to May. The only evidence
available for this potential trend is that of the three male whelks collected from the JR in
April, one of the contained the highest TBT concentration of any other JR male whelks.
The large increase in the variance of the TBT concentration in the OV males between
March and April could be a precursor to a larger increase in TBT concentration due to
increasing activity with warmer temperatures. Unfortunately, this potential trend cannot be confirmed since no males were collected during May.

**Life History of Female Veined Rapa Whelks, Based on Monthly TBT Concentration**

A compilation of all of the previously described trends and fluctuations in TBT body concentration of the females yields a possible general life history of the female whelks. In August, once egg laying has finished the whelks probably continue in a state of normal to high activity while they prepare for overwintering. During this period, their tissue concentration of TBT increases because they accumulate TBT from their food faster than they are able to degrade it. This phase is evident in the increase in TBT concentration from August to January. While there was one OV imposex female analyzed from December, since no other whelks were analyzed to validate the elevated TBT concentration, this whelk will not be discussed here. Once cold temperatures set in and the activity of the whelks is largely diminished, uptake of TBT is reduced enough that it is counteracted by elimination, which results in TBT concentrations remaining roughly constant from January through March. From March through May, warmer waters trigger increased feeding and respiration, which causes a increase in the TBT concentration of the whelks, since uptake overcomes elimination. Finally, once egg laying begins, the elimination of TBT through egg laying provides a sufficient amount of elimination that TBT concentrations decrease from May through August.

From January through April, the concentration of TBT in the JR males did not vary greatly. Male whelks from the OV region were only analyzed from two months, which limits the available information to relate the concentration of butyltins to life
history. If the annual trend is similar to the females, TBT concentration would increase during April and May because of increased activity. From March to April, the mean and variance of the TBT concentration in the OV males increased, but not significantly. The only data that suggests that the JR males will display a similar trend is that one of the three males collected during April contained the highest concentration of TBT of any of the JR males that were analyzed. The absence of males collected in May prevents the determination of a seasonal trend in male rapa whelks.

During February and March, the TBT concentration of the JR males was about 3 times that of the JR females. By April, the mean TBT concentration of the JR females was similar to that of the JR males. One explanation for this is that the females have a higher rate of uptake of TBT during this period. A higher uptake rate could occur if the females are eating a greater amount than the males, in order to prepare for egg laying. During breeding, both sexes must undergo advanced development of their gonads, but only the females invest a large amount of energy into the development of eggs. The eggs represent a substantial loss of energy when they are laid. To make up for this energy deficit females may eat more than males, and thus accumulate more TBT. Differential food consumption between sexes in the Spring would reduce the uptake of the males and help to explain why male whelks from several different age groups have similar TBT concentrations. Before the differences in male and female TBT concentrations can be fully explained, the feeding and breeding behavior as well as the energy budget of both males and females needs to be better understood.
Implication for Biomonitoring

Biomonitoring involves the analysis of animal tissue or a measurement of a physical expression of contamination that is representative of the state of pollution in a area. Biomonitors allow regulators to collect organisms from several different regions and by analyzing the organisms; they can determine the concentration of pollution. By collecting organisms from a wide range of sites, contaminated hot spots and annual trends in pollution can be identified. The complexity of organisms often complicates biomonitoring. One common assumption is that the tissue concentration of the animal reflects the concentration of the contaminant in the water (Laughlin, 1996).

Imposex has been used for several years as a bioindicator of TBT contamination. The degree of imposex development in the dogwhelk has been shown to be strongly related to the body concentration of TBT (Gibbs and Bryan, 1996). Furthermore, field surveys have found that the intensity of imposex increases with proximity to marinas and other TBT contaminated sites. Based on the current research, the degree of imposex in rapa whelks could only be used to indicate the concentration of DBT contamination in the imposex females and males. Further research is required to determine if, as is argued here, the DBT tissue concentration is a direct result of past TBT exposure. If this is the case then the cycling of butyltins in the organisms as well as the time that is required to develop a particular degree of imposex will also have to be determined, before biomonitoring is feasible.

Instead of using the degree of imposex, the muscle concentrations of TBT in the rapa whelk could be used to indicate local contamination; however, the seasonal shifts in
contamination will have to be anticipated. If female whelks are collected from one site in January and another site in May, the tissue concentrations will indicate the latter site is more contaminated when in reality the seasonal fluctuation in tissue concentration is the only difference. The life history of an organism and how this affects contaminant body concentrations must be well understood before it is used for biomonitoring purposes.

Future Research

This research has raised a number of new questions that require further study:

1) Does TBT exposure induce imposex in rapa whelks? Further evidence is required to solidly link TBT exposure to the degree of imposex. A controlled field experiment exposing rapa whelks to water containing a gradient of TBT contamination may help clarify the link.

2) Are the butyltin tissue concentrations of non-egg laying females similar to males? All of the whelks analyzed for this project were of reproductive age because collection methods have not yielded juvenile rapa whelks. The mechanism of maternal transfer of TBT from females to their eggs has been proposed as the reason that males generally have a higher concentration of TBT than females. If non-egg laying females possess similar TBT concentrations to males than the above argument will be stronger.

3) If TBT is the initiator of imposex, why are there imposex females and normal females that contain similar concentrations of TBT? The imposex females and normal females from OV contained similar concentrations of both TBT and DBT during all of the months these two groups were sampled. Unfortunately, the rarity of normal females in the field limited their collection from JR and the majority of those caught were in April
and May. May has been shown to be a time of heightened activity, perhaps leading to short-term elevation in TBT concentration and may not be sustained long enough for imposex development.

4) Are contaminants, other than TBT, acting to induce imposex in rapa whelks? Other contaminants in the aquatic environment have been shown to cause endocrine disruption and to induce imposex. Analysis of rapa whelk tissue for other contaminants may determine if tissue concentrations of other contaminants are related to the degree of imposex. TBT may be the dominant cause of imposex, while the actions of other contaminants intensify the effects of TBT.

5) Is the first mass of eggs (which contains the most TBT) less viable than later masses? C. Ware (2001) showed that hatch success of early and late egg masses was not significantly different; however, TBT has been shown to have many chronic effects. The long-term viability and reproductive health of whelks hatched from early and late egg masses is currently under investigation at VIMS.
CONCLUSIONS

1) Veined rapa whelks from the Chesapeake Bay are bioaccumulating both TBT and DBT in their muscle tissues.

2) There was a strong relationship between the tissue concentration of DBT and increased penis length in both the JR imposex females and males.

3) Males generally had higher concentrations of TBT and DBT than females.

4) Egg laying is a significant route of elimination of TBT for female rapa whelks.
LITERATURE CITED


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