Persistence of Tributyltin-Induced Imposex in Dogwhelks (Nucella lapillus) and Intersex in Periwinkles (Littorina littorea) in Atlantic Canada

Camille Coray\textsuperscript{1,3} and Shannon M. Bard\textsuperscript{1,2,*}

\textsuperscript{1} Department of Biology and \textsuperscript{2} Environmental Science, Dalhousie University, Room 820, Life Science Centre, 1355 Oxford Street, Halifax, NS B3H 4J1
\textsuperscript{3} Present address: Jacques Whitford, 115 Harrisville Blvd., Moncton, NB E1H 3T3

Imposex in gastropods such as dogwhelks (Nucella lapillus) is a sexual abnormality that can cause sterility through the superimposition onto females of male sexual characteristics such as a penis homologue and/or vas deferens. Intersex similarly affects periwinkles (Littorina littorea) and is characterized by a phenotypic disturbance of sex determination between gonad and genital tract. A major culprit in causing imposex and intersex in aquatic gastropods is tributyltin (TBT), which is primarily used as a biocidal compound in antifouling paints, but is also present in sewage and wastewater due to industrial use. Since 1989, TBT has been banned in Canada for use on boats under 25 m in length, and in 2003, the ban was extended to include vessels of any length. However, observations of persistent imposex and intersex in marine snails from Halifax Harbour, Nova Scotia, Canada, suggest that current TBT controls are ineffective to eliminate this environmental problem in active ports. Dogwhelk imposex incidence ranged from 19\% at the mouth of Halifax harbour to 100\% midharbour, while no dogwhelks were found at the harbour head. Dogwhelks from uncontaminated reference sites outside the harbour yielded no cases of imposex. Imposex frequency and Relative Penis Size Index decreased at some of the sites between 1995 and 2006, but imposex frequency increased from 65 to 100\% near a container pier, suggesting that TBT from antifouling paint on large (>25 m) vessels like container ships may still be a strong environmental concern in Halifax Harbour. Periwinkles can tolerate higher TBT exposure and can be found where dogwhelks have been extirpated because of TBT contamination. Intersex incidence ranged from 0\% outside the harbour to 100\% at the head of the harbour at sites where no dogwhelks were found. Intersex indices corresponded with imposex indices where dogwhelks were found, and were relatively high (>2.0) where dogwhelks were absent. Intersex rates and severity remained constant between 1996 and 2006. These data suggest that TBT-induced imposex may be at least partially responsible for the absence of dogwhelks in certain areas of Halifax Harbour. We also examined the impact of the loss of whelks, important predators for dominant mussels, on intertidal community structure and function. Due to the persistence of TBT in sediments and input of TBT into aquatic environments from sources other than vessel antifouling systems, current TBT controls may be of limited effectiveness in minimizing endocrine disruption and population decline in susceptible marine gastropods.

\textbf{Key words:} imposex, intersex, tributyltin, dogwhelks (Nucella lapillus), periwinkles (Littorina littorea), Halifax Harbour

\section*{Introduction}

Tributyltin (TBT) is best known for its use as a biocidal compound in antifouling paints designed to prevent biota from settling on ships' hulls, harbour and offshore installations, and fish-pen nets associated with aquaculture. TBT-based paints were hailed as a breakthrough for controlling growth of aquatic organisms on underwater surfaces and quickly replaced copper-based paints for use as an antifouling agent in the 1960s and early 1970s. TBT is also used as a preservative for wood, textiles, paper, and leather, as a slimecide for cooling systems within the pulp and paper industry and breweries, as stabilizing material in polyvinyl chloride pipes, and as a catalyst for synthesis of polyurethane foam and silicone rubber (Dubej and Roy 2003). Thus, in addition to the leaching of TBT from paints on immersed surfaces, TBT is released to the aquatic environment through municipal sewage, particularly when sewage treatment is lacking (Fent 1996).

Harmful effects of TBT on aquatic biota were first noted over a quarter century ago in Arcachon Bay, Western France (Alzieu et al. 1980). TBT exposure and accumulation were linked to oyster shell malformation, poor spatfall, and a near collapse of the local oyster farming industry (Alzieu et al. 1980). TBT contamination has since been correlated with a variety of detrimental effects in aquatic animals including endocrine disruption in gastropods (Matthiessen and Gibbs 1998), impaired immune function in Mytilus mussels (St-Jean et al. 2002a; St-Jean et al. 2002b), and immunotoxic and genotoxic effects on fish and other aquatic species (Patricolo et al. 2001; Tiano et al. 2001). TBT has also been noted for its toxic effects in humans, including impairment of natural killer cell function (Thomas et al. 2004). In TBT contaminated areas, TBT has been measured in every aquatic organism sampled, from lower trophic level phytoplankton and snails to higher trophic level fish, seabirds, sea otters, dolphins, whales, and even humans (Kannan and Falandysz 1997; Kannan et al. 1998; Tanabe et al. 1998).

* Corresponding author; shannon.bard@dal.ca
In 1982, France began limiting the use of organotin-based antifouling paints to vessels over 25 m in length (Alzieu 1991). France was followed over the years by Australia, the United States, New Zealand, South Africa, Hong Kong, and the European Union (Evans et al. 2000). Canada began limiting the use of TBT in antifouling systems to vessels over 25 m in length, and to aluminum vessels of any length under the Pest Control Products Act in 1989 (Canada Department of Agriculture 1989). In inland fresh waters across Canada, TBT was found at lower concentrations and less frequently in 1994 than in 1982 to 1985, but freshwater sediment samples showed TBT to be found at comparable concentrations and more frequently in 1994 than a decade earlier (Maguire 2000). Canada’s 1989 regulation had little effect in reducing TBT concentrations in seawater and sediments sampled almost a decade later (Chau et al. 1997). These findings are due to the high environmental persistence of TBT in sediments. After being released into the water column, TBT is degraded by the successive loss of its butyl groups to dibutyltin, monobutyltin, and inorganic tin. At levels of TBT contamination typical of contaminated surface waters, the half-life is 6 days to 4 months (Maguire 1987), but the half-life of TBT in sediments ranges from 360 to 775 days in surficial sediments to tens of years in anaerobic sediments (Dowson et al. 1996). A TBT half-life of 87 ± 17 years (± standard error of the mean) was measured in deep marine sediments sampled in the Saguenay Fjord, Quebec, Canada, a semi-enclosed marine system with sediment permanently submitted to sub-Arctic cold conditions (Viglino et al. 2004). Degradation rates of TBT increase as temperature and light intensity increase, and adsorption of TBT increases with a decrease in salinity (St-Louis 1999).

On 25 November 1999, the International Maritime Organisation adopted resolution A. 895(21), agreeing on a global prohibition of the application of organotin-containing compounds which act as biocides in antifouling systems on ships by 1 January 2003, and a complete prohibition of the presence of organotin compounds which act as biocides in antifouling systems on ships by 1 January 2008 (International Convention on the Control of Harmful Systems 2006). Adopting this resolution is a positive step toward eradicating what has been described as “perhaps the most toxic chemical that has ever been deliberately introduced into the aquatic environment” (Maguire 2000). However, TBT contamination may continue to be of concern in the aquatic environment due to its persistence and continued influx from sources such as polyvinyl chloride leachates (Maguire et al. 1993); water and sewage plants (Fent et al. 1991; Chau et al. 1992; Fent 1996; Voulvouli et al. 2004; Harrison et al. 2006), and wood preservation facilities (de Mora and Phillips 1997).

An important ecological effect of persistent TBT contamination in aquatic environments is a TBT-induced endocrine disruption termed “imposex” which can cause extirpation of certain aquatic snail populations. Imposes in gastropods is a sexual abnormality involving the superimposition onto females of male sexual characteristics such as a penis homologue and/or a vas deferens (Smith 1971). Imposes has been described in over 140 species of gastropods (Prosobranchia) worldwide (Omae 2003), and is thought to be caused by a disruption in the balance of steroid hormones (Matthiessen and Gibbs 1998). Field surveys and dose-response bioassays have shown TBT to induce imposex in dogwhelks (Nucella lapillus) at TBT levels of <0.5 ng of Sn per L in water (Bryan et al. 1987). Although aqueous copper sulphate and environmental stress have been suggested as potential variables contributing to the development of imposex in the wine-mouthed lepsiella (Lepsiella vinosa), a marine snail common to Australia (Nias et al. 1993), this claim remains unconfirmed. TBT is thus widely accepted as the predominant cause of imposex (Bryan et al. 1988), and a direct relationship has been pinpointed between TBT body burden and severity of imposex in N. lapillus (Gibbs et al. 1987; Barroso and Moreira 2002; Galante-Oliveira et al. 2006). In many gastropod species, the imposition of a penis and sperm duct appears to cause little interference with the reproductive activity of the affected female, but in neogastropod populations of the family Muricidae, including N. lapillus, the structure of the oviduct may be modified to the degree that reproduction is inhibited, causing population decline and eventual extirpation (Matthiessen and Gibbs 1998). Comparison of observations made in the late 1960s with those made in the mid-1980s along the coast of southwest England showed a decline, and, in some cases, the total disappearance of formerly abundant N. lapillus populations (Bryan et al. 1986). Surviving populations of N. lapillus surveyed in southwest England in 1985 were all affected by imposex, with those close to boating and shipping centers with high levels of TBT-use affected most severely (Bryan et al. 1986).

Intersex is a different TBT-induced virilisation phenomenon affecting periwinkles (Littorina littorea), and is characterized by a phenotypic disturbance of sex determination between gonad and genital tract (Deutsch and Brick 1993). Bauer et al. (1995) concluded that the threshold level of TBT in water for the development of intersex in L. littorea is 10 to 15 ng of Sn per L, in comparison with under 0.5 ng of Sn per L needed to induce imposex in N. lapillus (Bryan et al. 1987). L. littorea is thus well suited for monitoring in areas where TBT contamination is higher (>2.0 ng of Sn per L) because it can survive in areas where N. lapillus has been extirpated.

In 1995, all female N. lapillus examined in Portuguese Cove, Halifax Harbour, Nova Scotia, Canada, were affected by imposex, and areas within Halifax Harbour with apparently suitable habitat were lacking N. lapillus entirely (Prouse and Ellis 1997). In 1996, intersex was recorded in L. littorea surveyed at several sites within Halifax Harbour (Covert 1997). Intertidal diversity studies conducted in 2005 and 2006 (Coray and Bard 2006) revealed that N. lapillus is still absent from areas within Halifax Harbour with apparently suitable habitat, whereas L. littorea was observed at all sites.
The purpose of the present study was to determine the current incidence and severity of both imposex in *N. lapillus* and intersex in *L. littorea* at sites within and around Halifax Harbour. Current imposex and intersex measurements were compared to those made in 1995 (Prouse and Ellis 1997) and 1996 (Covert 1997) in order to assess changes that may have occurred due to the 1989 legislative ban on TBT-use on vessels under 25 m in length, and the 1999 ban preventing new coats of TBT containing paint from being applied on vessels of all lengths commencing 1 January 2003. The two indices of imposex (relative penis size [RPS] and the vas deferens sequence [VDS]) are strongly correlated with concentrations of tin as TBT in ambient seawater and *N. lapillus* tissues (Gibbs et al. 1987; Galante-Oliveira et al. 2006), and were used to establish gradients of TBT contamination in this study. In areas where *N. lapillus* were absent, intersex index (ISI) in *L. littorea* was used as a measure of TBT contamination (Bauer et al. 1995; Oehlmann et al. 1998).

**Materials and Methods**

**Study Area**

Eleven sites were surveyed in July 2006: nine within Halifax Harbour, Nova Scotia (Admiral’s Cove, Bedford Institute of Oceanography (BIO), Birch Cove, Container Pier, Dartmouth Cove, Dingle, Ferguson’s Cove, Herring Cove, and Pt. Pleasant), and two outside of Halifax Harbour, Nova Scotia (Duncan’s Cove and Long Cove Pond) (Fig. 1). Sites were chosen based on the following criterion: presence of suitable rocky intertidal habitat for *N. lapillus*, presence of mussels and barnacles which are a major food source for *N. lapillus*, and overlap with sites surveyed for intertidal diversity (Coray and Bard 2006).

**Measurements**

Presence or absence of *N. lapillus*, both juvenile and adult, was recorded at each site. At sites where *N. lapillus* was present in sufficiently high numbers (BIO [n = 43], Container Pier [n = 41], Ferguson’s Cove [n = 39] and Pt. Pleasant [n = 41]) (Fig. 1; Table 1), up to 43 adults (>20 mm shell length) were collected. Specimens were examined alive in the laboratory within 24 hours of collection and were not narcotized. Shell length was measured to the nearest 0.1 mm, and weight in shell was measured to the nearest 0.01 g. The shell was then cracked open with a mallet, and soft tissues were separated from the shell remains to measure weight out of shell (g). Penis length was measured to the nearest 0.1 mm under a binocular microscope equipped with an ocular micrometer. Mean female and male penile lengths (FPL and MPL, respectively) were used to calculate the Relative female-to-male Penis Size Index (RPSI) (RPSI = FPL/MPL x 100) for each site (Gibbs et al. 1987). VDS stages of females were assigned according to the scales described by Gibbs et al. (1987) and Oehlmann et al. (1998), and a VDS index (VDSI) was calculated as the mean of the VDS stages among assessed specimens at each site. Imposex frequency, the percentage of females affected by imposex at each site, was recorded as well as the male to female ratio.

Up to 42 adult *L. littorea* were collected from sites where *N. lapillus* were absent (Admiral’s Cove [n = 31], Birch Cove [n = 32], Dartmouth Cove [n = 42] and Dingle [n = 38]) or not abundant enough to sample (Herring Cove [n = 40] and Long Cove Pond [n = 40]) while 20 to 40 adult *L. littorea* were collected from sites where *N. lapillus* were present and abundant enough to sample (BIO [n = 29], Container Pier [n = 30], Ferguson’s Cove [n = 20] and Pt. Pleasant [n = 37]) (Table 2). Specimens were anaesthetized for two hours before examination in a 7% (weight per volume) magnesium chloride solution (in distilled water). Shell height was measured to the nearest 0.1 mm and weight in shell was measured to the nearest 0.01 g. The shell was then cracked open with a mallet and soft tissues were separated from the shell remains to measure weight out of shell (g). The ISI, or average intersex stage in a population (total number of intersex stages in a sample per number of analyzed females), was determined for each site based on criteria established by Bauer et al. (1995). Specimens exhibiting parasitic infection were excluded from analysis because parasitism can cause confounding modification of the genital system (Bauer et al. 1995). The male to female ratio was also recorded.

**Statistical Analysis**

Statistical analyses were conducted using the computer analytical package SPSS 14.0. For *N. lapillus*, a correlation matrix using Pearson correlation coefficients was created to examine correlations between snail size (shell length [mm], weight in shell [g], weight out of shell [g], weight of shell [g]) and penis length (mm) in both female and male specimens at each site. Linear regression
TABLE 1. Imosex data for dogwhelks (Nucella lapillus) at sites in and around Halifax Harbour, Nova Scotia, Canada in 2006

<table>
<thead>
<tr>
<th>Site</th>
<th>Date of survey (day/mo/yr)</th>
<th>No. of Specimens</th>
<th>Average shell length (mm) ± SD</th>
<th>FPL $^a$ (mm) ± SD</th>
<th>RPSI $^a$ (%)</th>
<th>Imosex frequency $^b$ (%)</th>
<th>VDSI $^a$</th>
<th>M/F ratio $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admiral’s Cove</td>
<td>22/07/06</td>
<td>none found $^c$</td>
<td>25.0 ± 4.2</td>
<td>0.7 ± 0.3</td>
<td>5.1</td>
<td>92</td>
<td>3.5</td>
<td>0.79</td>
</tr>
<tr>
<td>BIO</td>
<td>20/07/06</td>
<td>24</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birch Cove</td>
<td>22/07/06</td>
<td>none found $^c$</td>
<td>26.0 ± 4.0</td>
<td>1.0 ± 0.2</td>
<td>3.6</td>
<td>100</td>
<td>3.7</td>
<td>2.15</td>
</tr>
<tr>
<td>Container Pier</td>
<td>20/07/06</td>
<td>13</td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dartmouth Cove</td>
<td>22/07/06</td>
<td>none found $^c$</td>
<td>26.8 ± 3.0</td>
<td>0.0 ± 0.0</td>
<td>0</td>
<td>0</td>
<td>0.00</td>
<td>1.44</td>
</tr>
<tr>
<td>Dingle</td>
<td>21/07/06</td>
<td>none found $^c$</td>
<td>24.3 ± 1.8</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>50</td>
<td>1.1</td>
<td>0.50</td>
</tr>
<tr>
<td>Duncan’s Cove</td>
<td>21/07/06</td>
<td>9</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferguson’s Cove</td>
<td>21/07/06</td>
<td>26</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herring Cove</td>
<td>21/07/06</td>
<td>not enough found $^d$</td>
<td>25.2 ± 4.0</td>
<td>0.1 ± 0.2</td>
<td>0</td>
<td>19</td>
<td>0.5</td>
<td>0.58</td>
</tr>
<tr>
<td>Long Cove Pond</td>
<td>12/09/06</td>
<td>not enough found $^d$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. Pleasant</td>
<td>20/07/06</td>
<td>26</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ FPL, average female penis length; RPSI, Relative female to male Penis Size Index (= FPL/Average Male Penis Length x 100); VDSI, vas deferens sequence index; M/F ratio, ratio of males to females.

$^b$ Imosex Frequency = percentage of imposexed females in a population of dogwhelks.

$^c$ Suitable habitat and diet for N. lapillus was found at these sites.

$^d$ Three N. lapillus were observed at the Herring Cove site in the spring, but none were found on the date of sampling. Five N. lapillus were observed at the Long Cove Pond site in the spring, and only one was found on the date of sampling. It is unclear whether imposex is responsible for this lack of N. lapillus, or just natural variation in population numbers. It is possible that imposex is responsible for the low number of N. lapillus at the Herring Cove site due to a corresponding high rate of intersex in periwinkles surveyed there, but unlikely that imposex is responsible for the low number of N. lapillus at the Long Cove Pond site due a corresponding zero rate of intersex in periwinkles surveyed there.
TABLE 2. Intersex data for periwinkles (*Littorina littorea*) at sites in Halifax Regional Municipality, Nova Scotia, Canada in 2006

<table>
<thead>
<tr>
<th>Site</th>
<th>Date of survey (day/mo/yr)</th>
<th>No. of Specimens</th>
<th>Average shell height (mm) ± SD</th>
<th>Intersex frequency a (%)</th>
<th>ISI b</th>
<th>M/F ratio b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Admiral's Cove</td>
<td>22/07/06</td>
<td>19/22</td>
<td>16.4 ± 1.5</td>
<td>100</td>
<td>2.11</td>
<td>0.63</td>
</tr>
<tr>
<td>BIO</td>
<td>20/07/06</td>
<td>22/21</td>
<td>17.2 ± 1.4</td>
<td>89</td>
<td>0.41</td>
<td>0.32</td>
</tr>
<tr>
<td>Birch Cove</td>
<td>22/07/06</td>
<td>19/21</td>
<td>17.2 ± 1.5</td>
<td>89</td>
<td>0.41</td>
<td>0.32</td>
</tr>
<tr>
<td>Container Pier</td>
<td>20/07/06</td>
<td>21/9</td>
<td>25.0 ± 2.5</td>
<td>52</td>
<td>0.67</td>
<td>0.43</td>
</tr>
<tr>
<td>Dartmouth Cove</td>
<td>22/07/06</td>
<td>12/30</td>
<td>15.3 ± 1.3</td>
<td>100</td>
<td>2.10</td>
<td>2.50</td>
</tr>
<tr>
<td>Dingle</td>
<td>21/07/06</td>
<td>28/10</td>
<td>21.0 ± 1.5</td>
<td>82</td>
<td>1.46</td>
<td>0.36</td>
</tr>
<tr>
<td>Duncan's Cove</td>
<td>12/09/06</td>
<td>22/18</td>
<td>20.8 ± 2.3</td>
<td>0</td>
<td>0.00</td>
<td>0.82</td>
</tr>
<tr>
<td>Ferguson's Cove</td>
<td>21/07/06</td>
<td>12/8</td>
<td>23.9 ± 2.4</td>
<td>58</td>
<td>0.58</td>
<td>0.67</td>
</tr>
<tr>
<td>Herring Cove</td>
<td>21/07/06</td>
<td>27/13</td>
<td>25.3 ± 2.3</td>
<td>93</td>
<td>0.93</td>
<td>0.48</td>
</tr>
<tr>
<td>Long Cove Pond</td>
<td>12/09/06</td>
<td>19/21</td>
<td>2.08 ± 1.3</td>
<td>0</td>
<td>0.00</td>
<td>1.11</td>
</tr>
<tr>
<td>Pt. Pleasant</td>
<td>20/07/06</td>
<td>20/17</td>
<td>18.3 ± 1.3</td>
<td>15</td>
<td>0.15</td>
<td>0.85</td>
</tr>
</tbody>
</table>

aIntersex frequency = percentage of female *L. littorea* affected by intersex in a population.
bISI, intersex index; M/F ratio, ratio of males to females.

Analysis was also performed to test for correlations between male to female ratios and measurements of imposex (RPSI, VDSI, and imposex frequency) at each site. For *L. littorea*, a correlation matrix using Pearson correlation coefficients was created to examine correlations between female snail size (shell height [mm], weight in shell [g], weight out of shell, weight of shell [g]) and intersex stage. Linear regression analysis was also performed to test for correlations between male to female ratio and intersex measurements (ISI and intersex frequency) at each site.

Results

Imposex

Within Halifax Harbour, *N. lapillus* specimens were observed and collected at the BIO, Container Pier, Ferguson’s Cove, and Pt. Pleasant sites, and were not present at the Admiral’s Cove, Birch Cove, Dartmouth Cove, and Dingle sites (Fig. 1; Table 1). *N. lapillus* specimens were not collected at the Herring Cove site as only three animals were observed (Fig. 1; Table 1). Outside Halifax Harbour, sufficient numbers of *N. lapillus* were observed to permit collection at the Duncan’s Cove site, but, due to insufficient numbers (n = 6) at the Long Cove Pond site, no specimens were collected (Fig. 1; Table 1). Imposex was present in *N. lapillus* collected at all four sites surveyed in the harbour, but was absent at the reference site outside of Halifax Harbour (Fig. 1). Imposex frequencies in the harbour ranged from 50 to 100% with the highest (100%) measured at the Container Pier site; the VDSI in the harbour ranged from 0.5 to 3.7 with the highest (3.7) measured at the Container Pier site; and the RPSI in the harbour ranged from 0.0 to 5.1, with the highest (5.1) measured at the BIO site (Table 1). The male to female ratio ranged from 0.50 to 2.15 with the lowest (0.50) recorded at the Ferguson’s Cove site and the highest (2.15) recorded at the Container Pier site. No significant relationships were found between size of snail (shell length [mm], weight in shell [g], weight out of shell [g], or weight of shell [g]) and average male and female penis size at any site. No significant relationships were found between the male to female ratio and any measure of imposex (imposex frequency, RPSI, and VDSI).

Five sites in this study (BIO, Dartmouth Cove, Dingle, Container Pier, and Ferguson’s Cove) (Fig. 1) overlapped with sites that were previously surveyed for *N. lapillus* imposex in a 1995 study (Prouse & Ellis 1997). *N. lapillus* specimens were observed at only three of the five sites (BIO, Container Pier, and Ferguson’s Cove) (Fig. 1), both in 1995 and 2006. Imposex frequency has remained constant at the BIO site (92%), decreased from 95 to 50% at the Ferguson’s Cove site, and increased from 65 to 100% at the Container Pier site in just over a decade (Fig. 2). During that same period (11 years), the RPSI decreased from 10.8 to 3.1 at the BIO site, from 6.9 to 3.6 at the Container Pier site, and from 7.1 to 0.0 at the Ferguson’s Cove site (Fig. 3).

Fig. 2. Comparison of imposex frequency in dogwhelks (*Nucella lapillus*) surveyed at sites in Halifax Harbour, Nova Scotia, Canada in 1995 (Prouse & Ellis 1997) and 2006 (this work). Suitable habitat and diet for *N. lapillus* were found at these sites.
Discussion

In 2006, the four surveyed sites lacking *N. lapillus* within Halifax Harbour were located within the inner harbour in close proximity to marinas, shipyards, and docking facilities for large vessels (Admiral’s Cove, Birch Cove, and Dingle sites are located in areas where abundant recreational boating takes place. Despite current TBT bans, lack of enforcement increases the likelihood that TBT antifouling paint may be being used illegally on these smaller vessels. The Admiral’s Cove site and Birch Cove site are located in the Bedford Basin, a basin with a central depression of up to 70 m deep. Fine-grained, organic-rich sediments are deposited in all of the deep water areas of Bedford Basin (Buckley and Winters 1992; Buckley et al. 1995). TBT has a high binding capacity to finer particulate matter like that deposited in the Bedford Basin, and can persist for longer time periods and at higher levels under such conditions (Batley 1996). Sediment samples taken in 2002 near the Birch Cove site showed deepwater sediments in the Bedford Basin to contain 56.9 ng of TBT-Sn per g (dry weight [d.w.]), which is a higher TBT level compared to the value of 5.25 ng of TBT-Sn per g (dry weight [d.w.]) measured in 1996 near the Dartmouth Cove site.

Intersex

*L. littorea* was observed and sampled at all sites (Fig. 4). A total of 18 female specimens were excluded from morphological analysis due to parasitic infection: three from the Dingle site, seven from the Birch Cove site, and six from the Admiral’s Cove site. One male from the Dartmouth Cove site also exhibited parasitic infection. Intersex was present in *L. littorea* at all sites in the harbour (Admiral’s Cove, Birch Cove, BIO, Container Pier, Dartmouth Cove, Dingle, Ferguson’s Cove, Herring Cove, and Pt. Pleasant) and not present at the Duncan’s Cove and Long Cove Pond sites located outside the harbour (Fig. 4, Table 2). Intersex frequencies in the harbour ranged from 15 to 100% with the highest frequency (100%) occurring at two of the sites where *N. lapillus* specimens were absent (Admiral’s Cove and Dartmouth Cove) (Fig. 1), and intersex indices in the harbour ranged from 0.15 to 2.11 with the highest indices occurring at sites with 100% intersex frequency (Table 2). The male to female ratio ranged from 0.32 to 2.50 with the lowest (0.32) recorded at the BIO site and the highest (2.50) recorded at the Dartmouth Cove site (Table 2). No significant relationships were found between female snail size (shell height [mm], weight in shell [g], weight out of shell [g], and weight of shell [g]) and intersex index. No significant relationship was found between the male to female ratio and ISI.

Three sites (BIO, Dartmouth Cove, and Container Pier) (Fig. 4) overlapped with sites that were surveyed for *L. littorea* intersex in 1996 (Covert 1997). ISI measurements have not changed significantly at any of these sites in the last decade: from 0.44 to 0.41 at the BIO site, from 0.76 to 0.67 at the Container Pier site and from 2.06 to 2.10 at the Dartmouth Cove site (Fig. 5).
than that measured at Shearwater (42 ng of TBT-Sn/g), a naval base where three large vessels and over 100 pleasure craft dock (Carter et al. 2004) (Fig 6A). Tissue TBT concentration measured in mussels (Mytilus edulis) taken from the Bedford Yacht Club, near the Admiral’s Cove site in 2002 was 2.5 ng Sn per g (wet weight [w.w.]) (Carter et al. 2004) (Fig. 6B). Sediment levels of TBT can be measured at sites with relatively fine sediment, but organisms such as mussels and dogwhelks require habitat that includes coarser sand and solid rock. It is thus difficult to find sites where TBT concentrations can be measured in both sediment and animal tissue due to different substrate requirements. The Dartmouth Cove site is situated between the Dartmouth Marine Slips and the Dartmouth Shipyards, a docking and refurbishing facility for larger ships.

Ships serviced at the Dartmouth Shipyards were being painted with TBT as recently as late 2002. Concentrations of over 2,000 μg/g of TBT have been recorded in sandblasting grit used by Atlantic Canada shipyards to remove TBT coatings (Hennigar and Garron 1992), and TBT-containing paint chips are a by-product of sandblasting as well. Around active shipyards, debris containing TBT will enter the aquatic environment until the full ban on the use of TBT in antifouling systems is in effect in early 2008. Sediment samples taken in 2002 from the Dartmouth Marine Slips contained 1,985 ng of TBT-Sn per g (d.w.) and the TBT concentration measured in M. edulis sampled from Dartmouth Cove in 2002 was 1.4 ng of Sn per g (w.w.) (Carter et al. 2004) (Fig. 6). Although the Dartmouth Marine Slips closed in 2001 and TBT concentrations measured in 2002 [1,985 ng of TBT-Sn per g (d.w.)] were lower than those measured in 1994 (4,870 ng of TBT-Sn per g [d.w.]) and 1988 (6,500 ng of TBT-Sn per g [d.w.]) (Ernst et al. 1999), the 2002 levels were still considered to be at levels high enough to be considered hazardous to benthic organisms (Carter et al. 2004). The sites in question all contain suitable habitat for N. lapillus, including a food supply of mussels and barnacles, but their location near sources of historical and contemporary TBT contamination suggests that the absence of N. lapillus may be due to TBT-induced sterility, which lead to extirpation. The intersex survey results recorded for L. littorea at these sites are consistent with this hypothesis. Admiral’s Cove and Dartmouth Cove sites both had a 100% intersex frequency, and ISI measurements of 2.11 and 2.10 respectively; the Birch Cove site had an 89% intersex frequency and an ISI measurement of 2.00, and the Dingle site had an 82% intersex frequency and an ISI measurement of 1.46 (Fig. 2, Table 2). ISI values at all of the sites lacking N. lapillus in this study exceeded 1.0, which corresponds with a 1993 to 1996 investigation of coastal areas in Ireland, France, and Germany where N. lapillus populations were not found to survive in areas where L. littorea exhibited ISI values above 1.0 (Oehlmann et al. 1998).

Of the 5 sites surveyed where N. lapillus was observed (BIO, Container Pier, Duncan’s Cove, Ferguson’s Cove, Pt. Pleasant) (Fig. 1), the site with the highest incidence of imposex (100%) was located adjacent to a container pier (Container Pier) with a constant flow of ships over 2.5 m in length. Ships of this size are exempt from restrictive

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**Fig. 6.** Locations of sampling sites in Halifax Harbour, Nova Scotia, Canada for TBT. (A) Sites surveyed for imposex in Nucella lapillus and intersex in Littorina littorea in 2006 (this work) shown in relation to sites where TBT was analyzed in sediments in 2002 (Carter et al. 2004). (B) Sites surveyed for imposex in Nucella lapillus and intersex in Littorina littorea in 2006 (this work) shown in relation to sites where TBT was analyzed in Mytilus edulis tissue in 2002 (Carter et al. 2004). The relatively low levels of TBT tissue contamination found by Carter et al. (2004) may be partially due to the winter sampling period (December to January). Uptake of TBT tends to decrease in the late summer to winter, providing time for TBT to be metabolized to dibutyltin and monobutyltin, so that concentration of these metabolites increase compared to TBT in the tissues of contaminated animals during this time period (St.-Jean et al. 1999).
legislation on TBT passed in several countries in the 1980s, and the recent TBT ban affecting ships of all sizes will not be fully in place until 1 January 2008.

The next highest rate of imposex (92%) was observed at the BIO site, where large ships often dock. Fifty percent imposex was observed at a site adjacent to a major shipping lane (Ferguson’s Cove), and 19% imposex was observed at a site adjacent to a major municipal sewage outfall (Pt. Pleasant). No imposex was observed at the reference site (Duncan’s Cove), where no known current input or historical contamination of TBT is evident.

VDSI values were found to be as high as 3.7 near the Container Pier site and 3.5 near the BIO site. When the VDSI comes close to or exceeds 4.0 in a N. lapillus population, the population becomes endangered due to progressive female sterilization (Oehlmann et al. 1998). However, no sterile females were observed among the specimens sampled at these sites, and an abundance of N. lapillus egg cases indicates the reproductive capacity of these populations. Dispersal ability of N. lapillus is limited due to the lack of a planktonic larval stage, decreasing the possibility that N. lapillus sampled at these sites could have originated from other populations. TBT concentrations in M. edulis sampled at the Container Pier (South Container Terminal) and BIO sites in 2002 were 2.8 ng and 4.8 ng of Sn per g (w/w), respectively (Carter et al., 2004) (Fig. 6B).

Higher imposex frequencies corresponded with higher VDSI and RPSI values, demonstrating consistency in these measurements. Intersex survey results recorded for L. littorea at these sites (BIO, Container Pier, Duncan’s Cove, Ferguson’s Cove, Pt. Pleasant) (Fig. 3) were also consistent with the imposex findings. Intersex areas with higher ISI values correspond to imposex areas with higher VDSI and RPSI values, and no intersex was found at either of two reference sites (Duncan’s Cove, Long Cove Pond) (Fig. 3).

Two sites (Herring Cove, Long Cove Pond) (Fig. 1) yielded inconclusive results for N. lapillus. During intertidal diversity studies (Coray and Bard 2006), two N. lapillus specimens were observed at the Herring Cove site in June 2006, and one N. lapillus specimen was observed at the same site in August 2006. During these same intertidal diversity studies (Coray and Bard 2006), the following were observed at the Long Cove Pond site: two N. lapillus specimens in June 2006; three N. lapillus specimens, including N. lapillus egg cases, in August 2006; and one N. lapillus specimen in October 2006. Due to insufficient numbers, neither site could be included in the imposex survey. However, L. littorea at the Herring Cove site had a high intersex frequency (93%) and a high ISI value (0.93) (Table 2), suggesting that TBT contamination may be responsible for low numbers of N. lapillus at this site. Conversely, no cases of intersex were observed at the Long Cove Pond site, suggesting that low N. lapillus population numbers at this site are a product of natural variation in population size. Presence of N. lapillus egg cases at the Long Cove Pond site suggests a viable population of N. lapillus, whereas lack of N. lapillus egg cases at the Herring Cove site suggests the possibility of a nonviable N. lapillus population. Herring Cove exhibit an ISI of 0.93 and should L. littorea populations exhibit an ISI of 0.5 or more, it is likely that all of the females in a N. lapillus population will have become sterile (Oehlmann et al. 1998).

Herring Cove is a busy harbour town with heavy local boat traffic, and is located in the outer portion of Halifax Harbour near a major shipping lane. The Herring Cove site is also in close proximity to a large municipal sewage outfall. The Long Cove Pond site is not near any known shipping area, although there is minimal recreational boating and fishing activity in the vicinity. N. lapillus recruits were recently hatched individuals crawling from egg cases rather than free-swimming plankton. Because of their low mobility, colonization of sheltered areas by N. lapillus can be dependant on chance events like storms blowing individuals from other populations onto shore. Long Cove Pond is fairly sheltered, which may explain the low numbers of N. lapillus found here.

In 1995, Prouse and Ellis (1997) determined that, despite restrictions in place at that time, TBT contamination continued to be a widespread concern in eastern Canada. Halifax Harbour, Nova Scotia was included in their list of problem areas, indicated by results of imposex surveys. Results of this study suggest the same conclusion, even 16 years after initial bans on TBT-use on smaller vessels in Canada. A comparison of imposex data from 2006 (current study) with data from 1995 (Prouse and Ellis 1997) showed that little change had occurred in imposex frequency found in N. lapillus populations at three sites surveyed in both years (BIO, Container Pier, Ferguson’s Cove) (Fig. 2 and 3). The only drop in imposex frequency occurred at the Ferguson’s Cove site (from 95 to 50%), whereas imposex frequency at the BIO site stayed the same (92%), and imposex frequency at the Container Pier site increased (from 64 to 100%). Imposex frequency may have decreased at the Ferguson’s Cove site over the past decade due to some reduction in TBT-containing paints being used on ships hulls, although the total ban is not yet in place. Because Ferguson’s Cove is near a major shipping lane and is not directly adjacent to an area where large ships are docked, current input of TBT has likely decreased more in the last decade there in comparison with sites where ships sit for longer time periods. Many ships were likely given a fresh coat of TBT-containing paint in late 2002 before the total ban went into effect on 1 January 2003. This potential fresh input of TBT into the environment, especially where larger ships dock, may explain the increase in imposex frequency at the Container Pier site. TBT body burden is significantly correlated with RPSI (and VDSI) values (Barroso and Morcira 2002; Galante-Oliveira et al. 2006), making these indices a sensitive measure for detecting spatial or temporal changes in TBT levels. A significant decline in RPSI values at all three sites (BIO, Container Pier, and Ferguson’s Cove) (Fig. 1 and 3) thus suggests a decline in TBT contamination in these areas.

A link between TBT, areas of high boating activity, and masculinisation in L. littorea in Halifax Harbour was
noted in 1996 (Covert 1997). Results of the present study suggest the same conclusion, a decade later. In fact, no significant change in ISI values over time was observed at sites surveyed for intersex in L. littorea in 1996 and 2006 (BIO, Container Pier, Dartmouth Cove) (Fig. 4 and 5). These results suggest that TBT contamination at these sites has not yet dropped below levels sufficient to reduce ISI values in L. littorea populations. Recovery from TBT-induced effects may take longer to manifest in L. littorea populations than in N. lapillus populations as the lifespan of L. littorea can exceed ten years (Gonwanloch and Hayes 1926) compared with a lifespan of six years or less in N. lapillus (Fretter and Graham 1962). Both intersex and imposex are irreversible. Also, because L. littorea has a free-swimming planktonic larval stage, each site may have individuals recruited from a number of different populations, which means that ISI values observed at one site may not necessarily be indicative of TBT contamination solely at that site.

TBT contamination can be highly localized, with sediment concentrations measured in 2002 at sites within Halifax Harbour ranging from 56.9 to 46,267 ng of TBT-Sn per g (d.w.) with a mean of 9,962 ± 20,313 ng of TBT-Sn per g (d.w.) (Carter et al. 2004). The highest levels (46,267 ng of TBT-Sn per g (d.w.) were measured at the Halifax Shipyard (Fig. 6A), four orders of magnitude higher than TBT levels (3.2 ng of TBT-Sn per g (d.w.) measured at a reference site with negligible boating and mooring activity (Carter et al. 2004). The 2002 TBT concentrations represented an increase at the Shearwater, Synchorliff, and Halifax Shipyard sites (Fig. 6A) from concentrations measured in 1994 and 1988 (Carter et al. 2004). In 2003, TBT sediment levels at the Halifax Shipyard decreased from 46,267 to 13,400 ng of TBT-Sn per g (d.w.), but still remained among the higher concentrations found worldwide (Carter et al. 2004). Very high TBT sediment concentrations measured in both 2002 and 2003 indicate that the current risk to benthic organisms remains high within Halifax Harbour due to the persistence of TBT in sediments.

Despite persistent risk, some recovery has occurred in N. lapillus populations in Halifax Harbour over the past 11 years. Recovery is evidenced by a decrease in RPSI at three sites surveyed both in 1995 and 2006. However, areas with suitable N. lapillus habitat in the inner harbour are still lacking N. lapillus.

Several species of thaid gastropods, of which N. lapillus is one, have been shown to play an important predatory role in structuring rocky shore communities (Lubchenco and Menge 1978; Menge 1983; Menge 1995; Minchinton 1990). Predation by N. lapillus in the mid to low intertidal area has been shown to have a significant impact on barnacle mortality along the southern shore of Nova Scotia (Minchinton 1990); results of intertidal work (Coray and Bard 2006) conducted in the areas discussed in this study corroborated this by showing a significant negative correlation between presence of N. lapillus and percent cover of barnacles.

It is well documented that certain keystone species can completely alter the structure of an ecosystem through their presence or absence. As first introduced by Paine (1966, 1969), a keystone species is one whose presence is crucial in maintaining the organization and diversity of their ecological communities, and N. lapillus seems to play this role in intertidal communities in and around Halifax Harbour. A significant positive correlation was found between the presence of N. lapillus and the number of intertidal species (species richness) (Coray and Bard 2006). The four sites where dogwhelks were absent had the lowest average species richness (Admiral’s Cove [23], Birch Cove [25], Dartmouth Cove [22], Dingle [27]) of all 11 sites sampled (BIO [30], Container Pier [36], Ferguson’s Cove [37], Herring Cove [34], Point Pleasant [33], Duncan’s Cove [47], Long Cove Pond [45]) (Fig. 1). The presence of N. lapillus was also positively correlated with numbers of algal species (Coray and Bard 2006). Lack of a key predator can lead to dominance by its prey, such as mussels and barnacles, which reduces the amount of available substrate for settlement and growth of macrophytes. Sites with N. lapillus also had significantly lower numbers of the invasive green crab (Carcinus maenas), indicating that N. lapillus is enough of a keystone predator in the Halifax Regional Municipality intertidal area that its presence may help to control populations of this invasive competitor (Coray and Bard 2006). No historical data exists to document previous existence of N. lapillus at these sites. Re-colonization and potential changes in intertidal community structure, if N. lapillus did at one time populate these areas, will be revealed through future monitoring.

Decreases in the extent and severity of imposex have been reported in many areas where legislative bans on use of TBT on smaller vessels (<2.5 m) have been in effect for a decade or more: Australia (Rees et al. 2001), western Canada (Reitsema et al. 2002), Ireland (Minchin et al. 1995), northwest Brittany (Huet et al. 2004), the North Sea (Evans et al. 1991; Evans et al. 1996; Birchenough et al. 2002), and southwest England (Bryan et al. 1993). Some studies have reported significant recovery in the last 15 years in areas of small boat activity, but increases or no change in imposex in areas adjacent to shipping ports (Minchin et al. 1995; Reitsema et al. 2002). In 2001, Birchenough et al. (2002) reported recolonization of 14 of 23 (61%) sites in the North Sea where dogwhelks were absent in the 1980s. However, despite the same legislative ban of TBT being in place, increases in imposex severity and frequency have been reported in Portugal (Barroso and Moreira 2002) and Spain (Ramón and Amor 2001) over the last several years, indicating inefficacy of their 1987 ban. Illegal use of TBT on smaller vessels, and alternative sources of TBT such as industrial effluent, may be responsible for this observed trend. The observation that imposex is increasing in some areas despite legislative bans on TBT highlights the importance of continuing to monitor gastropod populations in high risk areas. Future imposex and intersex research should focus on detecting impacts of TBT inputs other than antifouling paints on ships hulls.
TBT levels ranging well above those needed to cause imposex in *N. lapillus* were found in untreated sewage influent (1,900 to 20,600 ng of TBT-Sn per L) and in treated sewage effluent (700 to 14,500 ng of TBT-Sn per L); these TBT levels were measured in samples collected from eleven Canadian sewage treatment plants in the early 1990s (Chau et al. 1992). Concentrations of TBT found by Fent (1996) in treated sewage effluent in Switzerland were also in the toxicity range of oysters and neogastropods. These high levels of TBT found in treated sewage effluent were then discharged directly into the aquatic environment. Even higher levels of TBT were found in untreated sewage effluent (Fent et al. 1991; Chau et al. 1992; Fent 1996). Halifax Harbour is fed by over 100 sewage outfalls, 39 of which are municipal, and together contribute a discharge of more than 135 million litres of untreated sewage daily (Halifax Harbour Cleanup Project 1993). Untreated sewage effluent may thus be carrying thousands of nanograms of TBT into the marine environment of Halifax Harbour every day. Although the rates and severity of imposex and intersex observed in Halifax Harbour could be explained by continued input of TBT from vessel antifouling systems and historical contamination from vessel antifouling systems, the potential input of TBT from other sources should not be overlooked.

**Conclusions**

Although interest in TBT contamination has declined since the global ban on TBT-use in vessel antifouling systems came into effect, there exists a continued need to fill gaps in knowledge regarding input of TBT into the environment from sources other than TBT-containing paint. This study showed that dogwhelk and periwinkle populations in Halifax Harbour are still exhibiting negative reproductive effects caused by exposure to TBT despite ongoing bans. Investigation into all pathways through which TBT may enter aquatic systems is crucial to establishing effective legislation. Effective legislation will minimize continued release of TBT into the aquatic environment and pinpoint areas of current and historical contamination other than those with obvious input from antifouling paints. Strong legislation coupled with stringent enforcement to limit TBT release, and continuing monitoring programs to track recovery from endocrine disruption, will permit re-colonization of *N. lapillus* in areas where it has disappeared, and help to improve the health of existing populations.

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