APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2005; **19**: 324–328
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.886

Speciation Analysis and Environment AOC

Comparison of the acute toxicity of tributyltin and copper to veliger larvae of *Nassarius reticulatus* (L.)

Ana Sousa[1]*, Luciana Génio[1], Sónia Mendo[1] and Carlos Barrosoi[1,2]

[1]Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal
[2]CESAM — Centro de Estudos do Ambiente e Mar, Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal

Received 30 October 2004; Accepted 2 December 2004

The ban on the use of tributyltin (TBT) is promoting an increasing use of copper as an active biocide in antifouling paints, with consequent rising levels of this metal in the environment. This study assesses the acute toxicity of copper and tributyltin to the larvae of the mollusc gastropod *Nassarius reticulatus*. Recently hatched veligers were exposed to nominal TBT-Sn concentrations of 0.9, 1.4, 1.9, 2.8, 3.8, 4.7 and 5.6 μg l^{-1} and nominal copper concentrations of 9.4, 23.4, 46.9, 70.3, 93.8, 117.2, 140.6 and 164.1 μg l^{-1} for up to 96 h, under static conditions (17 ± 1 °C and 33 ± 1 psu). The percentage of larval mortality was determined for each organometal/metal concentration and exposure time (1, 24, 48, 72 and 96 h). Both TBT and copper had a highly significant effect on larvae survival ($p < 0.001$) for all times of exposure, except for the first hour in the particular case of TBT. The lowest observed effect concentration for TBT-Sn decreased over time from 3.8 μg l^{-1} at 24 h to 1.9 μg l^{-1} at 96 h, whereas for copper it remained constant over time (46.9 μg l^{-1}). The median lethal concentration (LC_{50}) for TBT-Sn decreased from 4.87 μg l^{-1} at 24 h to 1.78 μg l^{-1} at 96 h, and the LC_{50} for copper decreased from 83.08 μg l^{-1} at 24 h to 58.84 μg l^{-1} at 96 h. TBT is far more toxic to *N. reticulatus* larvae than copper. However, owing to the higher copper environmental concentrations, the risk factors of the two biocides may approach each other. This stresses the need to find adequate substitutes for organotin biocides in future antifouling paints. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: gastropod; *Nassarius reticulatus*; larvae; antifouling paints; TBT; copper; acute toxicity; risk factor (RF)

INTRODUCTION

Tributyltin (TBT) compounds have been extensively used in the last four decades as biocides in antifouling paints, which caused a new pollution problem around the world. Owing to its high toxicity towards non-target organisms,[1] TBT applications have been regulated over recent years. This has promoted an increased usage of copper as the active biocide in antifouling paints, with a consequent increase in the level of this metal in the environment.[2] The masculinization of gastropod prosobranch females due to TBT, a phenomenon coined as imposex by Smith,[3] is probably the best known example of endocrine disruption caused by a pollutant. Imposex is already described for 180 different species around the world, and it may lead to female sterility at highly polluted sites.[4,5] A severe ecological impact may be expected in species that lack a planktonic stage in their life cycle, since if no recruitment of young specimens occurs from other places then the population will become extinct. This has happened, for instance, with the dog-whelk *Nucella lapillus* (L.) at many sites on Atlantic coasts.[5–7] It is generally assumed that populations of species with planktonic stages in their life cycle will not be severely affected by female sterilization, as the supply of new individuals is always assured from less-polluted places. However, larval stages are, in general, more sensitive than the adults,[8] and thus more research should be addressed regarding the effects of pollutants on the recruitment of prosobranch species at highly polluted sites. Most of the studies regarding the toxicity of metals towards mollusc larvae have been performed on bivalves, such as *Scrobicularia plana*,[9–11] *Crassostrea gigas*,[12–16] *Mytilus*

*Correspondence to: Ana Sousa, Departamento de Biologia, Universidade de Aveiro, Aveiro, Portugal.
E-mail: anasousa@bio.ua.pt
Contract/grant sponsor: University of Aveiro; Contract/grant number: 200230.

edulis[17,18] and *Mytilus galloprovincialis*.[12,14] Curiously, very few ecotoxicological studies are available regarding gastropod larvae.[19]

The netted whelk *Nassarius* (= *Hinia*) *reticulatus* (L.) is a common European prosobranch gastropod that is distributed from the Black Sea and the Mediterranean, north to Norway and into the western Baltic.[20] *N. reticulatus* has an indirect development: females lay capsules between spring and summer and after 2–4 weeks veligers hatch from the capsule to start a planktonic phase of 1–2 months before settling.[21–23] This species is affected by imposex throughout Europe,[24–28] and female sterility has been reported for highly polluted sites. The aim of the present study is to assess and compare the acute toxicity of TBT and copper on the survival of veliger larvae of *N. reticulatus* and to evaluate whether the recruitment of this species might be affected at places where pollution by these (organo) metals is high.

METHODS

Bioassay procedure

N. reticulatus capsules were collected at Ria de Aveiro (northwest Portugal) in September 2003. The capsules were mainly attached to the algae *Laurencia pinnatifida*, so those algae were brought to the laboratory and maintained at $17 \pm 1\,°C$ in 250 ml beakers containing permanently aerated natural seawater (33 psu (practical salinity unit), equivalent to permill). Each capsule was monitored daily under a stereo microscope and those 'close to hatch', i.e. containing veliger larvae with developed eyes, pigmentation, statocists and beating velum, were ripped and larvae were collected with a micropipette. The test was started by adding approximately 8–10 recent hatched larvae to each 2 ml well containing 1.5 ml of test solution. Each treatment, including the control, was replicated 12 times, yielding a total of *ca* 100 larvae. No food was supplied. All the material was subjected to a previous acid bath in order to prevent adsorption of toxic substances. Temperature ($17 \pm 1\,°C$) and salinity (33 psu) were maintained constant and no renewal of test solutions was performed during the experiment.

Numbers of live and dead larvae were counted every 24 h under a stereo microscope between the first hour and the fourth day of the experiment. Live larva were those actively swimming in the water or lying on the bottom but with beating of the velum.

Test solutions

TBT chloride ($C_{12}H_{27}ClSn$, Fluka) solutions were prepared with filtered natural seawater (TBT-Sn $< 0.03\,\mu g\,l^{-1}$) at nominal TBT-Sn concentrations of 0.9, 1.4, 1.9, 2.8, 3.8, 4.7 and $5.6\,\mu g\,l^{-1}$. To improve the solubility of TBT in seawater, the organic solvent dimethyl sulfoxide (DMSO) was used to add TBT into the test solutions. Therefore, two controls were used, one with seawater and the other with seawater plus DMSO. The DMSO control received the same quantity of solvent as the treatments ($150\,\mu l^{-1}$).

Copper ($CuSO_4 \cdot 5H_2O$; JMV Pereira, Lda.) solutions were prepared at nominal copper concentrations of 9.4, 23.4, 46.9, 70.3, 93.8, 117.2, 140.6 and $164.1\,\mu g\,l^{-1}$ with filtered natural seawater (copper $<0.8\,\mu g\,l^{-1}$) to which 0.001% HNO_3 had been added to increase metal solubility and reduce surface plating on vessel walls. Filtered natural seawater was used as a control.

STATISTICAL ANALYSIS

The mean percentage of dead larvae and standard deviation were calculated for each treatment at 1, 24, 48, 72 and 96 h and compared with the controls (DMSO for TBT and seawater for copper exposures). One-way analysis of variance (ANOVA) followed by a *post hoc* Student Newman Keuls (SNK) test ($\alpha = 0.05$) was performed in order to test the null hypothesis that contaminants had no effect upon larval survival. The transformation arcsin $\sqrt{x}$ was applied to the data prior to the analysis, and variances of data were checked by means of Cochran's homogeneity test before running the ANOVA.[29]

The 'no observed effect concentration' (NOEC) and the 'lowest observed effect concentration' (LOEC) values were determined from the larval mortality data after using ANOVA and the *post hoc* SNK test. NOEC was defined as the highest concentration causing no significant mortality on larvae, and LOEC was defined as the lowest concentration causing significant mortality of larvae.[30,31] The median lethal concentration LC_{50} values, i.e. concentrations causing 50% of mortality, and their confidence limits were calculated using probit analysis.[32] The risk factor (RF) was calculated as C_{est}/LOEC, where C_{est} is the mean TBT-Sn or copper concentration in estuaries.

RESULTS

There was no significant difference in mortality observed in the seawater or the DMSO controls up to 96 h (SNK test, $a = 0.05$). The average mortality in the control was always lower than 20%, except for 96 h where mortality reached 24.7% for TBT-Sn and 27.2% for copper. Mortality values below 30% in the control group are acceptable for a valid oyster embryo larval test according to ASTM protocols.[33]

TBT had a highly significant ($p < 0.001$) effect on the survival of *N. reticulatus* larvae for all times of exposure, except for the first hour (Table 1). A highly significant increase in mortality occurred at a TBT-Sn concentration as low as $1.9\,\mu g\,l^{-1}$ for 72 h onwards, compared with the control. Higher TBT-Sn concentrations caused significant effects for lower exposure times, e.g. $\geq 3.8\,\mu g\,l^{-1}$ induced mortality from 24 h onwards (Fig. 1). The estimated values for NOEC, LOEC and LC_{50} are shown in Table 1. The NOEC and the LOEC

Table 1. Parameters of one-way ANOVA testing the effects of TBT and copper on the percentage of dead larvae of *N. reticulatus* at different times of exposure and the respective NOEC, LOEC, LC_{50} and LC_{50} 95% confidence intervals. $^{***}p < 0.001$; ns: $p > 0.05$

	TBT-Sn (μg Sn l^{-1})					Copper (μg Cu l^{-1})				
Time (h)	*F*	NOEC	LOEC	LC_{50}	95% Cl	F	NOEC	LOEC	LC_{50}	95% Cl
1	1.55 ns	—	—	—	—	40.46***	23.4	46.9	102.70	73.15–132.07
24	53.57***	2.80	3.80	4.87	3.67–6.07	46.53***	23.4	46.9	83.08	28.24–137.41
48	110.05***	1.90	2.80	3.59	3.14–4.03	85.93***	23.4	46.9	80.83	53.64–108.40
72	146.73***	1.40	1.90	2.50	1.29–3.72	91.62***	23.4	46.9	66.50	59.38–74.00
96	105.02***	1.40	1.90	1.78	1.44–2.12	91.62***	23.4	46.9	58.84	35.45–82.29

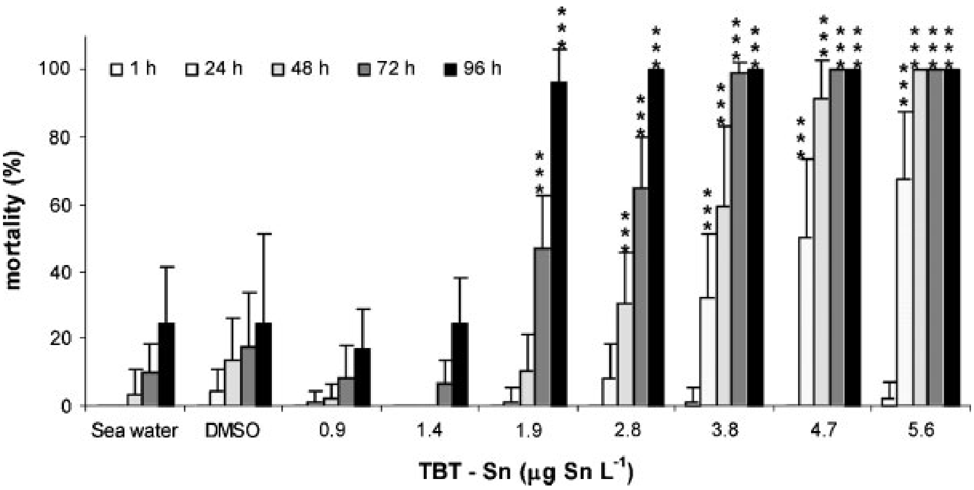

Figure 1. Percentages of dead *N. reticulatus* larvae in relation to different concentrations of TBT throughout the experimental period. Error bars represent the standard deviation for each concentration ($n = 12$). Concentrations where significant increasing mortality in relation to the DMSO control occurred are marked: $^{***}p < 0.001$.

decreased over the time of exposure: for TBT-Sn at 24 h they were 2.8 μg l^{-1} and 3.8 μg l^{-1} respectively, whereas for 96 h they were 1.4 μg l^{-1} and 1.9 μg l^{-1} respectively. The LC_{50} values also decreased with the time of exposure, from a TBT-Sn 4.87 μg l^{-1} (at 24 h) to 1.78 μg l^{-1} (at 96 h). Copper had a highly significant ($p < 0.01$) effect on the survival of *N. reticulatus* larvae for all times of exposure (Table 1). Copper toxicity remained insignificant up to 23.4 μg l^{-1} throughout the exposure time, but copper concentrations ≥46.9 μg l^{-1} had a highly significant ($p < 0.01$) effect on larvae mortality and 100% mortality was achieved for the highest concentration after 48 h of exposure (Fig. 2). NOEC and LOEC values for copper remained constant over time: 23.4 μg l^{-1} and 46.9 μg l^{-1} respectively. The LC_{50} values for copper varied from 102.70 (at 1 h) to 58.84 μg l^{-1} (at 96 h) (Table 1).

DISCUSSION

Few studies have measured the effects of the antifouling compounds TBT and copper on gastropod larval survival. This is the first one to report those effects on *N. reticulatus* veliger larvae.

TBT has a highly significant ($p < 0.001$) effect on the survival of recently hatched veliger of *N. reticulatus*. Our results show LC_{50} for TBT-Sn varying from 4.87 μg l^{-1} at 24 h to 1.78 μg l^{-1} at 96 h. The TBT-Sn LC_{50} (48 h) of 3.59 μg l^{-1} obtained in the current study compare with those reported for acute toxicity tests with gastropod and bivalve larvae. For gastropod larvae of *Thais clavigera, Haliotis discus discus* and *Haliotis madaka* the reported TBT-Sn LC_{50} values (48 h) are 2.29 μg l^{-1}, 2.21 μg l^{-1} and 0.49 μg l^{-1} respectively.[19] For the bivalve larvae of *Mytilus edulis, Crassostrea virginica* and *Mercenaria mercenaria* the TBT-Sn LC_{50} values (48 h) are 0.90 μg l^{-1}, 1.62 μg l^{-1} and 0.68 μg l^{-1} respectively.[8,34] These values are very high, however; if one were to extend the time of exposure of the tests, then lower effective concentrations would probably be found. For instance, the TBT-Sn LC_{50} in *M. edulis* larvae varies drastically from 0.90 μg l^{-1} for 24 h to 0.04 μg l^{-1} for 15 days.[8–18]

The LC_{50} and LOEC obtained in the current study are much higher than the environmental quality standards (EQSs) for TBT-Sn in the UK and the USA of 8.2×10^{-4} μg l^{-1} and 4.1×10^{-4} μg l^{-1} respectively,[35,36] and above TBT-Sn concentrations in water generally found in the open waters of estuaries

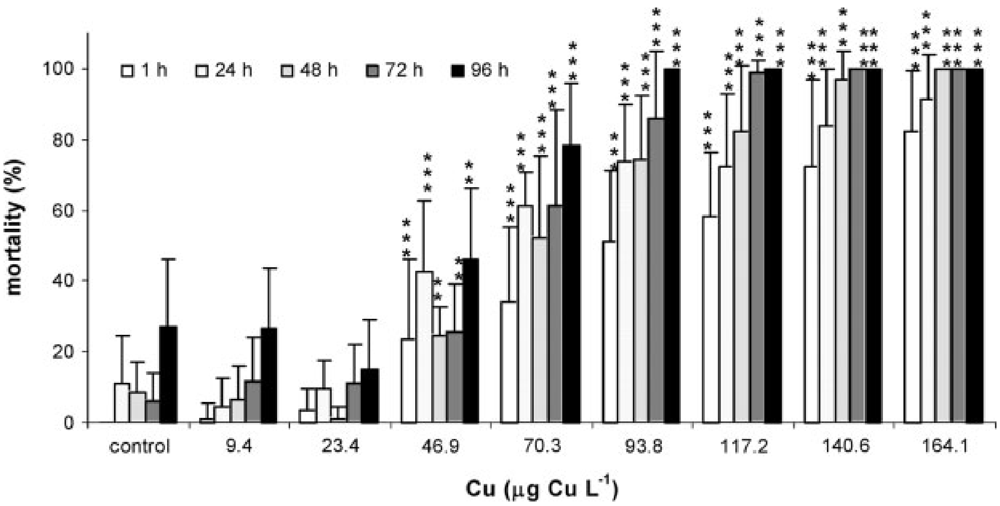

Figure 2. Percentages of dead *N. reticulatus* larvae in relation to different concentrations of copper throughout the experimental period. Error bars represent the standard deviation for each concentration ($n = 12$). Concentrations where significant increasing mortality in relation to the seawater control occurred are marked: $^{**}p < 0.01$; $^{***}p < 0.001$.

of between 4.0×10^{-3} and 4.0×10^{-2} μg l^{-1};[37] they are also higher than the TBT-Sn concentrations reported for dockyards and ports, which are commonly 0.10 to 0.20 μg l^{-1},[38] and they can occasionally reach 0.96 and 1.15 μg l^{-1}.[39] Thus, the present TBT-Sn concentrations observed in the environment do not affect the survival of *N. reticulatus* larvae within the first 96 h after hatching, since the RF is very low; e.g., considering a mean TBT-Sn concentration in estuaries of 4.0×10^{-2} μg l^{-1} and the 96 h LOEC (1.9 μg l^{-1}) gives an RF $\approx$ 0.021.

Regarding copper toxicity, our results show copper LC_{50} values varying from 102.70 μg l^{-1} (at 1 h) to 58.84 μg l^{-1} (at 96 h). The copper LC_{50} for 48 h obtained in this study (80.83 μg l^{-1}) is in the same range as values reported for gastropod, coral and bivalve larvae, such as *M. edulis* larvae, with a copper LC_{50} (at 48 h) of 84 μg l^{-1}.[30,31,40–44] Reported mean concentrations for total dissolved copper in harbours, estuaries and open sea regions from Europe are, according to Hall and Anderson,[45] 1.53 μg l^{-1}, 1.49 μg l^{-1} and 0.68 μg l^{-1} respectively. Copper levels between 2 and 6 μg l^{-1} have been reported[46] along the Portuguese coast, and exceptionally very high levels were observed in northwest Spain, e.g. in Ria Pontevedra (Galicia) with copper concentrations as high as 190 μg l^{-1}.[31,47] EQSs for copper in seawater are 5 μg l^{-1} in the UK and 2.9 μg l^{-1} in the USA and Denmark. In Europe, the Oslo and Paris Commission (OSPAR) set copper concentrations of 0.1–1.0 μg l^{-1} to be used as a guide to identify areas for further monitoring.[48] The lowest LC_{50} and LOEC values obtained for copper in the current study (58.84 μg l^{-1} and 46.9 μg l^{-1} respectively) are much higher than the EQS and the mean copper concentrations reported prior to the TBT ban. Considering a mean copper concentration of about 1.5 μg l^{-1} in estuaries, the RF for the larvae of this species is as low as 0.031. Nevertheless, if copper concentrations reported for Galician waters[47] are considered, then the risk factor approaches unity at several locations. In fact, copper environmental concentrations are expected to increase due to the growing use of copper-based antifouling paints, as has already been noticed in Arcachon Bay, where an increase in copper content in oysters harvested in a zone located in the vicinity of marine and mooring areas was registered between 1979 and 1991.[2] Moreover, the higher leaching rate of copper from self-polishing paints (25.0–40.0 μg $cm^{-2}day^{-1}$) relative to TBT (1.5–4.0 μg $cm^{-2}day^{-1}$)[49] contributes to the rise in copper environmental levels.

The present study reveals that TBT is far more toxic to *N. reticulatus* larvae than copper, a tendency also reported by several workers[41,50,51] for different larval species and different endpoints. The RF obtained for TBT and copper assuming pre-TBT-ban concentrations of both contaminants and acute exposure of up to 96 h are similarly very low for recently hatched larvae of *N. reticulatus*. Nevertheless, it is important to note the increasing tendency in copper levels as a result of its usage as a substitute for TBT in antifouling paints.[2,48,49] Moreover, for some locations there is already an important ecological risk associated with this contaminant, as noted by Beiras and Albentosa[44] for commercial bivalve populations in certain areas of the Galician coast.

Acknowledgements

This work was partly supported by the University of Aveiro through a Masters Grant (grant no. 200230).

REFERENCES

1. Alzieu C. *Ecotoxicology* 2000; **9**(1–2): 71.
2. Claisse D, Alzieu C. *Mar. Pollut. Bull.* 1993; **26**: 395.
3. Smith BS. *Proc. Malacol. Soc. London* 1971; **39**: 377.

4. Horiguchi T, Cho H, Shiraishi H, Shibata Y, Monta M, Shimizu M. *Bul. Coast Oceanogr.* 2000; **37**: 89 (in Japanese).
5. Gibbs PE, Bryan GW. TBT-induced imposex in neogastropod snails: masculinization to mass extinction. In *Tributyltin: Case Study of an Environmental Contaminant*, de Mora SJ (ed.). Cambridge University Press: Cambridge; 21–61.
6. Barroso CM & Moreira MH. *Mar. Pollut. Bull.* 2002; **44**: 480.
7. Ruiz JM. Quintela M, Barreiro R. *Mar. Ecol. Prog. Ser.* 1998; **164**: 237.
8. Thain JE. The acute toxicity of bis(tributyltin) oxide to the adults and larvae of some marine organisms. ICES Paper Cm 1983/E: 13 (mimeograph). International Council for the Exploration of the Sea, Copenhagen.
9. Ruiz JM, Bryan GW, Gibbs PE. *J. Exp. Mar. Biol. Ecol.* 1995; **186**: 53.
10. Ruiz JM, Bryan GW, Gibbs PE. *Mar. Biol.* 1995; **124**: 119.
11. Ruiz JM, Bryan GW, Gibbs PE, Wigham GD. *Mar. Environ. Res.* 1995; **40**: 363.
12. Geffard O, His E, Budzinski H, Seaman M, Garrigues P. *C. R. Acad. Sci. Ser. III. Sci. Vie* 2001; **324**: 1149.
13. Beiras R, Fernandez N, Bellas J, Besada V, Gonzalez-Quijano A, Nunes T. *Chemosphere* 2003; **52**: 1209.
14. His E, Seaman MNL, Beiras R. *Water Res.* 1997; **31**: 351.
15. His E, Heyvang I, Geffard O, de Montaudouin X. *Water Res.* 1999; **33**: 1706.
16. Labare ML, Coon SL, Matthias C, Weiner RM. *Appl. Environ. Microbiol.* 1997; **63**: 4107.
17. Lapota D, Rosenberger DE, Platter-Rieger MF, Seligman PF. *Mar. Biol.* 1993; **115**: 413.
18. Beaumont AP, Budd MD. *Mar. Pollut. Bull.* 1984; **15**: 402.
19. Horiguchi T, Imai T, Cho HS, Shiraishi H, Shibata Y, Morita M, Shimizu M. *Mar. Environ. Res.* 1998; **46**: 469.
20. Graham AFRS. Molluscs: prosobranch and pyramidellid gastropods. In *Synopses of the British Fauna*, 2nd edn, Kermack DM, Barnes SK (eds). The Linnean Society of London and the Brackish-Water Science Association: London, 1986; 186–189.
21. Tallmark B. *Mar. Ecol. Prog. Ser.* 1980; **3**: 51.
22. Fretter V, Graham A. *British Prosobranch Molluscs, Their Functional Anatomy and Ecology*. Dorset Press: Dorchester, 1994; 522.
23. Barroso CM, Moreira MH. *J. Mar. Biol. Assoc. U. K.* 1998; **78**: 1233.
24. Stroben E, Oehlmann J, Fioroni P. *Mar. Biol.* 1992; **113**: 625.
25. Bryan GW, Burt GR, Gibbs PE, Pascoe PL. *J. Mar. Biol. Assoc. U. K.* 1993; **73**: 913.
26. Barreiro R, Gonzáles R, Quintela M, Ruiz JM. *Mar. Ecol. Prog. Ser.* 2001; **218**: 203.
27. Barroso CM, Moreira MH, Gibbs PE. *Mar. Ecol. Prog. Ser.* 2000; **218**: 221.
28. Barroso CM, Moreira MH, Bebiano MJ. *Mar. Ecol. Prog. Ser.* 2002; **230**: 127.
29. Zar JH. *Biostatistical Analysis*, 3rd edition. Prentice-Hall International: Upper Saddle River, NJ, 1996.
30. Reichelt-Brushett AJ, Harrison PL. *Mar. Pollut. Bull.* 2000; **41**: 385.
31. Femandez N, Beiras R. *Ecotoxicology* 2001; **10**: 263.
32. Finney DJ. *Probit Analysis*. Cambridge University Press: Cambridge, 1971.
33. *Standard Guide for Conducting Static Acute Toxicity Tests Starting with Embryos of Four Species of Saltwater Bivalve Molluscs*. American Society for Testing and Materials: Philadelphia, PA, 1998; section E72494.
34. Roberts MH. *Bull. Environ. Contam. Toxicol.* 1987; **39**: 1012.
35. Abel R. European policy and regulatory action for organotin-based antifouling paints. In *Organotin: Environmental Fate and Effects*, Champ MA, Seligman PF (eds). Chapman & Hall: London, 1996; 27–54.
36. Ambient aquatic life water quality criteria for tributyltin (TBT). EPA-822-B-02-001, US EPA, Washington, DC, 2002.
37. Bryan GW, Gibbs PE. Impact of low concentrations of tributyltin (TBT) on marine organisms: a review. In *Metal Ecotoxicology: Concepts and Applications*, Newman MC, McIntosh AW (eds). Lewis Publishers: Ann Arbor, MI. 1991; 323–361.
38. Batley G. The distribution and fate of tributyltin in the marine environment. In *Tributyltin: Case Study of an Environmental Contaminant*, de Mora SJ (ed.). Cambridge Environmental Chemistry Series No. 8. Cambridge University Press: Cambridge, 139–166.
39. Prego R, Cobelo-Garcia A. *Environ. Pollut.* 2003; **121**: 425.
40. Ross KE, Bidwell JR. *Arch. Environ. Contam. Toxicol.* 2001; **40**: 489.
41. Negri AP, Heyward AJ. *Mar. Environ. Res.* 2001; **51**: 17.
42. Ramachandran S, Patel TR, Colbo MH. *Ecotoxicol. Environ. Saf.* 1997; **36**: 183.
43. Worboys MA, Leung KMY, Grist EPM, Crane M. *Mar. Pollut. Bull.* 2002; **45**: 92.
44. Beiras R, Albentosa M. *Aquaculture* 2003; **230**: 205.
45. Hall LM, Anderson R. *Mar. Pollut. Bull.* 1999; **38**: 207.
46. Quality status report 2000: region IV—Bay of Biscay and Iberian coast. OSPAR Commission, London, 2000; 1–134.
47. Beiras R, Femandez N, Bellas J, Besada V, Gonzalez-Quijano A, Nunes T. *Chemosphere* 2003; **52**: 1209.
48. Matthiessen P, Reed J, Johnson MS. *Mar. Pollut. Bull.* 1999; **38**: 908.
49. Omae I. *Chem. Rev.* 2003; **103**: 3431.
50. Ruiz JM, Bryan GW, Wigham GD, Gibbs PE. *J. Mar. Bio. Assoc. U. K.* 1996; **76**: 829.
51. His E, Beiras R, Quiniou F, Parr ACS, Smith MJ, Cowling MJ, Hodgkiess T. *Water Res.* 1996; **30**: 2822.

