Sensitivity to cadmium along a salinity gradient in populations of the periwinkle, *Littorina littorea*, using time-to-death analysis

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Received 26 November 2002; received in revised form 4 September 2003; accepted 25 September 2003

Abstract

In this study, we assessed the combined effect of Cd concentration and salinity, on Cd uptake and mortality rate of *Littorina littorea*, collected along a salinity and pollution gradient in the Western Scheldt estuary (The Netherlands). Animals kept at their field salinity levels were exposed to three Cd concentrations (i.e. 10, 40 and 320 μM), while animals kept in 10 μM Cd were subjected to five salinity treatments (i.e. 15, 20, 25, 30 and 35‰). Mortality was recorded every 24 h and Cd body burdens were measured with ICP-AES. Time-to-death data were analysed via Cox proportional hazard models, including the co-variables “site-Cd treatment” in the Cd experiment and “site-salinity treatment” in the salinity experiment. “Cd-treatment” and “field-salinity” affected mortality rates significantly in the Cd experiment, such that the mortality risk increased by 2.3 times when salinity was lowered from 35 to 15‰, while it decreased by 19.7 times when Cd dropped from 320 to 10 μM. “Site” did not significantly affect the mortality risk in the salinity experiment but affected time-to-death via its interaction with the “salinity-treatment”. Generally, mortality did not occur at a given threshold Cd tissue level, but changed over time and treatments, in function of the site. The results demonstrate the importance of the animals’ environmental history and illustrate the usefulness of time-to-death analyses in ecotoxicological experiments.

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Keywords: Cadmium; Cox proportional hazard model; *Littorina littorea*; Salinity

1. Introduction

Since estuaries, and coastal zones, in general, are major sites for urban and industrial development, they may be considered as waste fields for many pollutants, including heavy metals (e.g. Lam et al., 1997; Kasuba and Rozgaj, 2000; Suzuki et al., 2001).

Although heavy metals are natural constituents of the aquatic environment and furthermore, some metals are essential for living organisms, they may pose a threat to estuarine communities, when they occur in elevated concentrations (Lorenzon et al., 2000). However, the toxicity of a metal to aquatic organisms is not directly proportional to its total concentration in the environment, but strongly depends on its chemical speciation (e.g. Blust et al., 1995; Nair and Robinson, 2001). Indeed, a considerable body of evidence indicates that the bio-availability of a metal is not just...
function of its total concentration, but rather depends on its free ion activity (e.g. Blust et al., 1992; Jackson et al., 2000; Nair and Robinson, 2001 and references therein), which is affected by factors such as salinity and complexation capacity (e.g. Forbes, 1991; Bjergaard and Depledge, 1994; Hall and Anderson, 1995; Blaudez et al., 2000; Jackson et al., 2000; Heugens et al., 2001; Philip, 2001). In addition, estuarine organisms have to preserve their functional integrity across the existing salinity gradients. This, requires certain physiological adjustments to maintain the composition of the intracellular environment under control and different strategies exist to achieve this (i.e. ion and osmo-regulators versus ion and osmo-conformers). These physiological adjustments also have an effect on the exchange of ions and water with the environment and these changes may also have an effect on metal uptake and accumulation kinetics. Thus, understanding the effects of estuarine processes such as changes in salinity on metal uptake and accumulation requires a combination of chemical and physiological principles (e.g. Depledge and Rainbow, 1990; Rainbow, 1997; Wilgust and Jones, 1998; Roast et al., 2002). Moreover, metal levels that are sublethal to organisms living at optimal conditions may become lethal at suboptimal conditions, as the susceptibility to additional stressors increases when animals approach their environmental tolerance limits (Heugens et al., 2001). Thus, environmental factors do not only affect metal uptake and accumulation, but may also influence physiological condition and tolerance, shaping the distribution and abundance of the estuarine species in a direct and indirect way (e.g. Day et al., 1989; Levinton, 1995).

Among estuarine organisms, molluscs have frequently been used in monitoring programmes, as they generally have a wide salinity tolerance enabling them to penetrate far upstream, occur in high densities and are known to ingest considerable amounts of metals in an apparently unregulated fashion (e.g. Langston and Zhou, 1987; Mouneyrac et al., 1999; Evans et al., 2001). It has also been shown that organisms living near their lower salinity limits, generally contain higher metal levels compared with organisms living at higher salinities (e.g. Phillips, 1977; Bryan et al., 1983; De Wolf et al., 2000). This pattern has also been observed in the Scheldt estuary (The Netherlands), which has been ranked among the most heavily polluted estuaries in the world for both the dissolved and the particulate metal phase. Its current situation has improved, especially with respect to the dissolved metal phase, but particulate Cd levels remain high compared to other estuaries and are still 10 and 50 times higher than in unpolluted seas and oceans (Bayens, 1998). A clear Cd tissue concentration gradient was observed in the periwinkle Littorina littorea (Linnaeus, 1758) living along the Scheldt estuary, which coincided with an upstream increasing particulate and dissolved Cd concentration and a downstream increasing salinity gradient (De Wolf et al., 2000). Shells of downstream animals were significantly larger, than those of animals collected more upstream (De Wolf et al., 2001a), while densities decreased upstream as well (personal observation). The less favourable living conditions upstream (i.e. low salinity, high Cd pollution), were suggested to account for these observations, since they might increase the mortality rate, resulting in less and younger (i.e. smaller) specimens or might decrease the growth rate of L. littorea, as more energy would have to be allocated towards stress resisting processes.

The periwinkles of the Scheldt estuary therefore, present and interesting case to assess the combined effects of changes in heavy metal exposure and salinity on the tolerance of an estuarine invertebrate to metal toxicity. Differences in tolerance should not only depend on differences in metal bio-availability, bioaccumulation pattern or physiological condition, along the salinity and pollution gradient, but also depend on the degree in which the organisms are acclimatised or adapted to the site specific conditions, each with their own salinity and Cd pollution history. In particular, the study aimed to demonstrate possible differential effects of the environmental history (e.g. position occupied along the salinity and pollution gradient) on the Cd accumulation and sensitivity of L. littorea.

2. Methods and materials

2.1. Cadmium experiment

In order to analyse the effect of Cd on the mortality rate of L. littorea, living at different salinity regimes along the Western Scheldt estuary, we conducted the
following experiment. On 2 December 2000, L. littorea were collected at three sites along the Western Scheldt estuary (Fig. 1). These sites included: Westkapelle (35 ‰), Ellewoutsdijk (25 ‰), and Hansweert (15 ‰). Salinities were measured upon collection using a refractometer (Atago) with an accuracy of ±0.5 ‰. At each site, 200 animals were collected in the mid-littoral zone from a rocky surface. Animals were transported to the laboratory, and maintained for 5 days at 15 °C in plastic jars to allow evacuation of the gut. A series of 12 aquaria were prepared, of which the first four contained 35‰, the second four 25‰ and the last four 15‰ of artificial seawater (201; Marinemix: Wiegandt GmbH and Co). The animals collected in Westkapelle, Ellewoutsdijk and Hansweert were randomly assigned to the first, the second and the third group of four aquaria, respectively. The animals in each set of four aquaria were exposed to either 0, 10, 40 or 320 μg/g dry weight (HgCd) of total Cd, added as CdCl₂ · H₂O (Merck). Hence, each aquarium contained 50 animals, descending from a particular sampling site, kept in 20 l of artificial seawater, at a particular total Cd concentration (i.e. either 0, 10, 40 or 320 μM), and at a salinity that corresponded to their field salinity. Animals were kept submerged by placing them in plastic 15 × 15 × 15 "open-maze" containers at the bottom of the aquarium. The experiment lasted for 13 days and mortality was recorded every 24 h. Mortality was defined as a failure to respond to probing with forceps. Throughout the experiment, the periwinkles were not fed, while they were kept at 15 °C under full-spectrum light in a 12 h light:12 h dark regime.

2.2. Salinity experiment

In order to determine the effect of salinity on the mortality rate of L. littorea, descending from different salinity areas, the following “salinity experiment” was conducted. On 16 December 2000, L. littorea were collected at three sites along the Western Scheldt estuary (i.e. Westkapelle, Ellewoutsdijk and Hansweert). At each site, 200 animals were collected in the mid-littoral zone from a rocky surface. Similarly to the experiment outlined above, the animals were transported to the lab, where they were kept for 5 days at 15 °C in plastic jars to allow evacuation of the gut. Ten aquaria were each filled with 201 of artificial seawater, of which the first five did not contain Cd,
while the last five were set at 10 μM of Cd, added as CdCl₂·H₂O. The artificial seawater in both controls and Cd treatments was set at either 15, 20, 25, 30 or 35‰. After depuration, 20 animals of each sampling site were randomly assigned to the different treatments. Hence, each aquarium contained three groups of 20 individuals descending from three different sampling sites, kept in 20 l of artificial seawater, set at a specific salinity (i.e. either 15, 20, 25, 30 or 35‰) and Cd concentration (i.e. either 0 or 10 μM). As outlined above, animals were kept submerged by placing them in a plastic “open-maze” container at the bottom of the aquarium. The experiment lasted for 11 days and mortality was recorded every 24 h. Other exposure conditions and handling were outlined above.

2.3. Metal analysis

To determine the tissue cadmium concentration in L. littorea, during the cadmium and salinity experiment, Cd was measured upon mortality of the animals, recorded over 24 h time intervals. When mortality occurred, the tissue of the death animal was dried to a constant weight at 60°C. Individual soft tissues were subsequently digested in a microwave oven, adding a mixture (5:1) of HNO₃ (70%) and H₂O₂ (30%), following the protocol as described by Blust et al. (1988). Digested samples were frozen at −20°C until further analysis. Cadmium concentration was measured in the digested soft tissues of each death animal by inductively coupled plasma atomic emission spectrophotometry (ICP-AES), using a Varian Liberty Series II spectrometer (De Wit and Blust, 1998). Analytical efficiency was checked using standard reference material (Mytilus edulis, CRM 278R) from the Community Bureau of Reference (BCR), digested and analysed in the same way as the samples. Field soft tissue metal levels were measured by collecting an additional 10 individuals at Westkapelle, Ellewoutsdijk and Hansweert, on 2 December 2000, and subjecting them, after 5 days of depuration, to the same metal analysis as outlined above.

2.4. Statistical treatment

Mortalities in both the cadmium and salinity experiment were treated as censored data and analysed via the Cox proportional hazard model, expressed as:

\[ h(t) = h_{\text{ref.}}(t) e^{-x\beta} \]

where \( h(t) \) is the hazard of an individual at time \( t \), \( h_{\text{ref.}}(t) \) the baseline hazard at time \( t \), \( x \) a matrix of predictor variables or co-variates and \( \beta \) is a vector of regression coefficients that estimate the influence of each co-variate on the shape of the survival curve, or its hazard (Newman and Aplin, 1992). This model is not based on any assumptions concerning the nature or state of the underlying survival distribution. The model assumes that the underlying hazard rate is a function of the independent variables (i.e. co-variates) that were included (Newman and Aplin, 1992). Site [i.e. Westkapelle (35‰), Ellewoutsdijk (25‰) and Hansweert (15‰)] and Cd-treatment (i.e. 0, 10, 40 and 320 μM) were included as co-variates in the cadmium experiment, whereas site and salinity treatment (i.e. 15, 20, 25, 30 and 35‰) were chosen in the salinity experiment. An overall \( \chi^2 \) value was estimated for each Cox proportional hazard model (i.e. cadmium and salinity experiment), while the significance of the co-variates’ contribution to the deflection of the baseline hazard rate was calculated via a Wald statistic, tested against a \( \chi^2 \) distribution.

Accumulated Cd tissue levels, measured upon mortality, were analysed in both the Cd and salinity experiment by means of a three-way analysis of variance (3-ANOVA). In the Cd experiment, the fixed factors “site”, “time” and “cadmium-treatment” were contrasted, whereas “site”, “time” and “salinity”, were contrasted in the salinity experiment. A significance level of 5% was used throughout. All analyses were performed with the software package Statistica (Statsoft, 2000).

3. Results

3.1. Cadmium experiments

Time-to-death data from the Cd experiment were analysed via a Cox proportional hazard model of
Table 1

Results of the Cox proportional hazard survival analysis, for censored data from the Cd experiment, contrasting the co-variates "site" and "Cd-treatment".

<table>
<thead>
<tr>
<th>Co-variates</th>
<th>( \beta )</th>
<th>Standard error</th>
<th>Wald statistics</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>-0.0412</td>
<td>0.0066</td>
<td>38.6940</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cadmium</td>
<td>0.0096</td>
<td>0.0005</td>
<td>450.8092</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Model: \( \chi^2 = 445.9621; \text{d.f.} = 2; P < 0.0001 \)

which the results are summarised in Table 1. The survival curve of the hypothetical “average” individual, of which each co-variates (i.e. salinity/Cd treatment) is equal to the average value of that variable for the entire set of individuals, differs significantly from the survival curve generated by the fitted model, as shown by the log likelihood comparison of the null (i.e. all \( \beta \) estimates = 0) and the fitted model (Table 1; \( P < 0.0001 \)). Hence, at least one of the co-variates (site, Cd), must be significantly related to the mortality of *L. littorea* in this experiment. Indeed, when considering the \( \chi^2 \) values for the individual parameter estimates (i.e. \( \beta_{\text{site}}, \beta_{\text{Cd}} \)), it is clear that both co-variates affect the mortality rate of *L. littorea* significantly (Table 1).

The negative sign associated with \( \beta_{\text{site}} \) indicates that time-to-death increases downstream, where salinities are higher. In contrast, the positive sign associated with \( \beta_{\text{Cd}} \) indicates that time-to-death increase with decreasing Cd concentrations. Finally, based on the parameter estimates of the Cox proportional hazard model, we are able to estimate the relative mortality risks for *L. littorea*, exposed to any salinity and Cd level or combination of both. For instance, the relative mortality risk of *L. littorea*, living at 15‰, lies 2.3 times higher than that of its con-specifics living at 35‰, while the relative mortality risk is increased by 19.8 times when animals are exposed to 320, instead of 10 \( \mu \)M of Cd. Consequently, an animal kept at 15‰, and an ambient Cd concentration of 320 \( \mu \)M, has a mortality risk increase of 45.5 (i.e. \( 2.3 \times 19.8 \)) compared with an animal living at 35‰ and 10 \( \mu \)M of Cd. These risk assessments are graphically illustrated in the mortality plots (Fig. 2). Cumulative mortality increases with increasing ambient Cd levels, irrespective of the salinity conditions (i.e. 35, 25 or 15‰). However, within each Cd treatment, mortality decreases with increasing salinity levels. Hence, the highest cumulative mortality rate is found in the

![Fig. 2. Cumulative mortality plots for periwinkles kept at their field salinity level (i.e. Wenskapelle: 35‰; Ellewoutsdijk: 25‰; Hanseweert: 15‰), and varying Cd levels (i.e. 0, 10, 40, 320 \( \mu \)M of Cd). Cd levels between brackets represent the bio-available Cd fraction after correction for Cd speciation and ionic strength, calculated after Blust et al. (1992).](image)
Hansweert population (i.e. 15‰) kept at 320 μM of Cd.

Compared to the average field Cd levels measured in the soft tissues of *L. littorea* from Westkapelle, Ellensoustedijk and Hansweert (0.155 ± 0.036, 0.395 ± 0.147 and 1.166 ± 0.623 μg/g dry weight, respectively), it is clear that *L. littorea* accumulates high Cd levels, before it actually dies (Fig. 3). The average Cd tissue level measured in *L. littorea* from Hansweert, exceeds for instance its field level by 147 times, when kept for 192 h at 320 μM of Cd. Possible differences in Cd accumulation levels upon mortality, between the different sites, time intervals and Cd treatments are summarised in a three-way ANOVA...
Significant accumulation differences are found over time, between sites and Cd treatment (Table 2; all \( P < 0.0001 \)). However, Cd tissue level differences in both Cd treatment and time, are site dependent, as indicated by the significant time \( \times \) site and Cd-treatment \( \times \) site interactions (Table 2; both \( P < 0.0001 \)) and illustrated by the time \( \times \) site interaction plots (Fig. 3). Indeed, periwinkles do not die at a given particular Cd tissue concentration, as Cd tissue levels vary, both within and between the different time intervals and Cd treatments, which are clearly site dependent (Fig. 3). Nevertheless, a critical Cd body residue can be given (i.e. 48.38 ± 29.82 \( \mu g/g \) dry weight), below which no mortality occurs, irrespective of time, salinity and/or Cd conditions. Moreover, the Cd tissue level variation that exists, for instance within each site, between the different Cd treatments, though significant, appears rather small if the Cd exposure range is taken in to account as well. Indeed, maximum and minimum Cd tissue levels differ only by a factor 2, 3 and 3 within Westkapelle, Ellewoutsdijk and Hansweert, respectively, despite they are exposed to a Cd treatment that ranges over a factor 32 (Fig. 3).

### 3.2. Salinity experiments

The results of the Cox proportional hazard model for the salinity experiment are given in Table 3. The log likelihood of the null model differs significantly from the log likelihood of the fitted model (Table 3; \( P < 0.0001 \)), indicating that mortality is related to at least one of the co-variates (site, salinity). The \( \chi^2 \) values for \( \beta_{\text{site}} \) and \( \beta_{\text{salinity}} \) suggest that only the co-variate salinity has a significant effect on time-to-death (Table 3; \( \beta_{\text{salinity}} : P < 0.0001 \)). The positive sign associated with \( \beta_{\text{salinity}} \) (Table 3), indicates that time-to-death decreases as salinity decreases. Clearly, salinity has an effect, as illustrated in the cumulative mortality plots, but there is also a "site \( \times \) salinity" interaction effect (Fig. 4). Mortality increases with decreasing salinity levels in Westkapelle and Ellewoutsdijk only. Whereas

<table>
<thead>
<tr>
<th>Co-variate</th>
<th>( \beta )</th>
<th>Standard error</th>
<th>Wald statistics</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>-0.0008</td>
<td>0.0081</td>
<td>0.0081</td>
<td>0.9212</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.0436</td>
<td>0.0096</td>
<td>20.6506</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Model: \( \chi^2 = 21.6973; \text{d.f.} = 2; P < 0.0001 \).
the cumulative mortality rate in _L. littorea_ was the lowest for the 35‰ salinity treatment in the Westkapelle and Ellewoutsdijk sample (0 and 25%, respectively, after 264 and 240 h, respectively), it reached 95% after 216 h of exposure at 35‰ in the Hansweert sample (Fig. 4). Moreover, at the end of the salinity experiment (i.e. 264 h of exposure), the 35‰ salinity treatment had the highest cumulative mortality rate in the Hansweert population (95%), followed by the 20, 15, 30 and 25‰ salinity treatments (80, 75, 75 and 40%, respectively) (Fig. 4).

Possible differences in Cd accumulation levels upon mortality, between the different sites, time intervals and salinity treatments are summarised in a 3-ANOVA table (Table 4). Significant accumulation differences are found over time, between sites and salinity treatments (Table 4; all P < 0.0001). However, the current results are not straightforward to interpret, as all three- and two-way interactions, except for the time × salinity interaction, are significant as well (Table 4). Nevertheless, when interpreting the Cd accumulation plots at each site, for the different salinity treatments a general trend may be described (Fig. 5). Cadmium levels, measured upon mortality, generally increase over (1) time and (2) salinity treatment, irrespective of the site that is considered.

### 4. Discussion

As shown in the Cd experiment, mortality in _L. littorea_ (1) decreases with decreasing ambient Cd levels, irrespective of the salinity conditions, while it (2) increases within each Cd treatment, when salinity is lowered from 35 to 25 and 15%. These findings may be explained in terms of a salinity effect, which has a strong effect on Cd uptake and toxicity (e.g. Blust et al., 1992; Rainbow et al., 1993; Jackson et al., 2000). Indeed an increase in salinity results, among others, in an increase in ionic strength and osmolarity and calcium and chloride concentration. The latter results in the formation of cadmium chloride species that are of much lower toxicity than the free Cd²⁺ ions. The increase in ion strengths decreases the activity coefficients of the metal species and measurements with a Cd²⁺ ion selective electrode and modelling showed that the free Cd²⁺ ion activity represents only 0.65, 1.21 and 2.57% of the total Cd that is present in (inorganic) seawater of 35, 25 and 15‰, respectively (Blust et al., 1992). Since Ca²⁺ and Cd²⁺ may be taken up by the same transport systems (e.g. Elmanara and Bussieres, 1997; Philp, 1999; Blaudez et al., 2000) the competition for uptake between both ions will be salinity dependent (e.g. McLusky et al., 1986; Tendengren et al., 1988; Bjerregaard and Depledge, 1994; Hougens et al., 2001). The extent to which Ca²⁺ affects the Cd uptake varies among species, but is nonetheless considerable in _L. littorea_ (Bjerregaard and Depledge, 1994). Indeed, Bjerregaard and Depledge (1994) showed that cadmium uptake in periwinkles at 30‰ salinity was 38% of the uptake at 10% salinity. Seventy-six percent of this decrease was attributed to the calcium effect and 24% to the remaining salinity effects. The free cadmium ion activity at a salinity of 30‰ is about 24% of that at a salinity of 10%. Thus, it appears from these results, taken into account the calcium effect, that the change in free cadmium ion activity does not translate in an equally strong effect on cadmium uptake in the periwinkles. This implies that either other cadmium species can be taken up and/or that changes in ion and osmoregulatory performance counteract the cadmium speciation effect.
Fig. 5. Cd tissue levels, expressed as µg/g dry weight, measured upon mortality at 24 h time intervals.
Changes in the osmolarity of the exposure solution prompts the organism to adjust its osmoregulation and the osmotic behaviour is characterised by passive tolerance to large changes in cell volume due to osmotic water uptake or release (Todd, 1964; Rumsey, 1973; Taylor and Andrews, 1988). The volume of the extracellular fluid is conserved at the expense of intracellular fluid during salinity acclimation. To maintain iso-osmolality with the environment salts move between the extracellular fluid and external solution, while water moves between the extracellular and intracellular fluid to dilute or concentrate extracellular osmolytes (i.e. K+ and free amino acids). Intra- and extracellular fluids of acclimated Littorina are essentially iso-osmotic with the external medium and the ion composition of the extracellular fluid closely resembles that of the exposure solution down to about 50% seawater, but becomes hyperosmotic in more dilute environments (Todd, 1964). Thus, the main changes during a transfer from high to low salinity is uptake of water and a corresponding increase in intracellular volume to achieve iso-osmolality with the extracellular fluid and the environment. This process occurs within hours and therefore the organisms used in the current experiments can be considered to be fully acclimated to the experimental salinity conditions. Thus, for the same cadmium uptake rate, an organism acclimated to a low salinity would, on a wet weight basis, have a low Cd2+ ion activity, the Cd2+ and osmoregulatory effects, mortality in Ellewoutsdijk (25%) and Hansweert (15%) should be much higher, compared to the mortality observed in Westkapelle (35%). Indeed, if we correct for the Cd speciation effect, and consider the mortality profiles in terms of the free Cd2+ ion activity, we note that mortality decreases with decreasing Cd2+ ion levels. However, this decrease should drop by a factor 2 with each salinity transition within each Cd treatment. This is certainly not the case in the 320 and 40 μM Cd treatment, and can only partially explain the 10 μM mortality profiles. Moreover, it is clear that the discrepancy between the observed and expected mortality becomes even larger, when we would correct for the Ca2+ and osmoregulatory effects as well.

Interestingly, mortality does not occur at a given threshold Cd tissue concentration, but rather changes over time and Cd treatment, and is site dependent. Although periwinkles from Westkapelle have the lowest mortality rates for each of the considered Cd treatments, they generally die at a lower Cd tissue concentration, compared with their conspecifics from Hansweert and Ellewoutsdijk. Similarly, as mortality within each salinity treatment decreases with decreasing Cd levels, the Cd tissue concentration upon mortality decreases as well. The fact that less Cd is bio-available at higher salinities, should in principle not affect the amount of Cd that periwinkles take in before they die. Indeed, if metal detoxification rates are similar, it merely means that it would take longer at higher salinities before the lethal Cd tissue concentration is reached. Hence, it is suggested that periwinkles from Westkapelle are less tolerant, despite that they have a better survival in this experimental set-up. This result can be explained in terms of the periwinkles environmental history (e.g. Hall and Anderson, 1995; Tendengren et al., 1999). Indeed, periwinkles from Ellewoutsdijk and Hansweert live at low salinities and elevated ambient Cd levels and may thus be acclimated or locally adapted to these conditions (De Wolf et al., 2000). If so, we expect periwinkles from Westkapelle to be at a disadvantage when faced with similar living conditions. This is, indeed, shown in the Cd experiment, where periwinkles from Westkapelle have a higher mortality rate compared with those from Hansweert, when kept at similar Cd2+ activity (i.e. Westkapelle at 10 μM and Hansweert at 40 μM of Cd). A similar result is found in the salinity experiment. At an ambient Cd concentration of 10 μM, mortality increases dramatically in the Westkapelle group when salinity is lowered from 35 to 30, 25, 20 and 15‰. With the highest and steepest mortality rate at 10 μM of Cd and 15‰, Westkapelle periwinkles are the worst performers when exposed to the poorest living conditions. These latter results can not only be attributed to ion and osmoregulatory problems (e.g. Huni and Aravindan, 1985; Souza and Moreira, 1987; Tendengren and Kautsky, 1987), since none (i.e. 20, 25, 30 and 35‰) or a very small proportion of the Westkapelle animals died in the controls (i.e. 15‰, mortality 10%). It is
clear, therefore that the periwinkles descent or environmental history (i.e. acclimation, adaptation and/or maternal effects) affects the (1) mortality profiles and the (2) intersite Cd uptake differences, observed in both the Cd and salinity experiment.

In addition to the intersite differences in Cd uptake, we also observed uptake differences within each site, between the different Cd and salinity treatments. Indeed, Ellewoutsdijk animals kept in the Cd experiment for 168 h at 40 μM of Cd, accumulate three times less Cd before they die, than when they are kept for the same time at 320 μM of Cd (i.e. 56.17 and 139 μg/g dry weight, respectively). However, as stated, this variability range seems rather small when compared with the Cd exposure range. Hence, these results demonstrate that Cd toxicity expressed in terms of tissue levels, shows much less variation than when it is expressed on an exposure concentration basis within the timeframe of the experiments. Similar to the Cd exposure experiment, comparable differences in Cd uptake are found within each site among the different salinity treatments. Indeed, animals from Westkapelle held at 25‰ and 10 μM of Cd, die after 72 h with a Cd tissue concentration of 27.63 μg/g dry weight, even though they are able to withstand Cd tissue levels up to 61.49 μg/g dry weight, when kept for the same exposure time at 15‰.

Thus, it appears that periwinkles from Hansweert and Ellewoutsdijk are acclimated and/or adapted to the reduced salinity and/or increased Cd exposure conditions in which they live. Interestingly, genetic structuring has been found in this animal along the Scheldt estuary as well (De Wolf et al., 2001b). Based on the expression of its esterase loci, the polluted and lowest saline sites, clustered together in a multi-dimensional scaling plot, discriminating from the clean and more saline sites (De Wolf et al., 2001b). This structuring needs to be confirmed with DNA markers, as protein expression may be prone to gene regulation (Berger et al., 1975; Mazon et al., 1998). However, irrespective from the mechanisms which may be at work (i.e. adaptation, acclimation and/or genetically or environmentally determined maternal effects), this study illustrates the importance of an animals environmental history in understanding bioaccumulation and toxicity patterns.

This is confirmed by the Cox proportional hazard analysis. The strength of this analysis lies in the fact that it can identify individual and/or combinations of important factors and relate them mathematically to a particular risk. We could for instance calculate the mortality risk for L. littorea, living in Hansweert compared with Westkapelle. This risk is 2.3 times higher in Hansweert if we only consider the salinity differences between both sites. One can assess additional risks related to the input of Cd, and consider these risks along the estuary taking salinity into account as well. Time-to-event techniques are more powerful than classical concentration–effect methods, such as the LC50 test, since they (1) consider the time to respond for each individual, instead of the proportion of all exposed individuals that respond by the end of the exposure period and (2) generate more data, yielding more statistical power, making it easier to study and accurately model co-variate effects. Nonetheless, they are rarely implemented in ecotoxicological studies (Newman and Aplin, 1992) although they are widely used in other scientific disciplines, such as medicine (e.g. Detre et al., 1994; Sundquist et al., 2002) veterinary medicine (Bailey et al., 1999; Brandt et al., 1999), and socio-economics (e.g. Portugal and Addison, 1995; Osler et al., 2002).

5. Conclusion

The experimental and statistical approach enabled us to pin point environmental factors that are important for the survival of L. littorea along the western Scheldt estuary and relate them to relative mortality risks that correspond to any particular situation in the field (i.e. combination of salinity and Cd concentration). In addition the results indicate that the periwinkles descent or environmental history both affects (1) the Cd uptake and (2) mortality rate. Indeed, periwinkles descending from Cd polluted areas with low ambient salinity levels seem to be acclimated and/or adapted, as they perform better (i.e. tolerate higher Cd levels), compared with animals from less polluted areas, when faced with high Cd and low salinity levels.

Acknowledgements

The authors would like to thank Marcel Selens (RUCA) for performing the ICP-AES analyses and Harry Van Paeschen (KBIN) for the drawings. This
References


