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Assessing spill risks and impact from Hazardous and Noxious Substances (HNS): Are standard toxicity data enough?

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ABSTRACT 2017-262

Over recent decades there has been an increase in the shipping of chemicals at sea with a subsequent increase in the risk of incidents involving hazardous and noxious substances (HNS). Typically, during HNS spills and during scenario planning the impact upon marine organisms is estimated using spill model predictions of time-weighted average (TWA) or peak concentration compared to a predicted no effect concentration (PNEC) derived from laboratory based constant exposure toxicity studies. This paper evaluates two marine transported chemicals, aniline and benzalkonium chloride, and compares their toxicity during standard (constant) and brief (spill-profiled) exposures.

Toxicity was evaluated for the copepod *Tisbe battagliai*, the red algae *Ceramium tenuicorne* and the brown algae *Fucus vesiculosus*. Benzalkonium chloride (BAC) and aniline exhibited toxicity in similar concentration range with EC_{50}s typically 1-2 mg l^{-1} range during constant exposures. When brief or spill-profiled exposures were considered, contrasting effects for the two test chemicals were shown across the range of test species. Exposure to BAC resulted in significant effects to *T. battagliai* (the most sensitive species to this chemical) after 1-hour exposure to a concentration of 5 mg l^{-1} but for aniline recovery of the test organisms still occurred in exposures of a few hours at concentrations up to 300 mg l^{-1}.

Thus, a spill of a chemical with a specific mode of toxicity results in greater effects following relatively brief exposures, and exposed organisms show little or no recovery. In such cases impacts might be expected to be more extensive and persistent.

In conclusion, for chemicals with specific modes of toxic action brief exposures, and possibly limited spill profile studies may be useful to support chemical spill risk assessment. Considerations of concentration exposure profiles during spills may also be relevant to take account of when considering oil spill impact plans and predictions.

INTRODUCTION

Over the past four decades, world seaborne shipments grew at an annual average rate of 3 per cent, rising from 2.6 billion tons in 1970 to 8.4 billion tons in 2010 (United Nations Conference, UNCTAD 2013). The UK marine environment has some of the busiest shipping lanes and ports in the world and a large offshore oil & gas industry. During 2013 the Marine Accident Investigation Branch recorded for non UK commercial vessels 8 collisions of solid bulk cargo vessels and 7 involving liquid bulk cargoes in UK waters (MAIB Annual Report, 2013). These types of incidents illustrate the potential risk to the marine environment from accidental spills of chemicals and oil.

Hazardous and Noxious Substances (HNS) are defined as any substance other than oil, which if introduced into the marine environment is likely to create hazards to human health, to harm living resources and other marine life as well as to damage amenities and/or to interfere with other legitimate uses of the sea (IMO, 2000). It is recognised that HNS transported at sea provide an extremely broad range of marine spill scenarios due to the 1000's of types transported in bulk through UK waters and their widely varying fate and effects. As such, there is a need to improve our knowledge of the marine hazards/risks associated with the highest priority chemicals so as to provide better advice during marine incidents. In particular, there is a requirement to better understand environmental effects when biota are exposed to realistic concentration profiles which would occur during spills and could include high peak concentrations and repeated exposures.

Data for the top 100 European ports over years 2002 - 2004 showed aniline to be ranked 24/100 in terms of tonnage bulk handled (over 300,000 tonnes) for this period (HASREP, 2005). Based on physico-chemical properties the Bonn Agreement, 1994 categorised HNS substances into a number of behaviour categories. Under this categorisation aniline is classed as a floater dissolver (FD).

Aniline was therefore selected as a test chemical in this study because: 1) it is known to be frequently transported in large quantities (Neuparth et al., 2011), 2) dissolves in water, and 3) is toxic.

Benzalkonium chloride (BAC) was also chosen for assessment as it is one of the most important quaternary ammonium compounds, it is used for a wide variety of applications, is a high production chemical that is likely to be transported in bulk quantities, it readily dissolves and is toxic (Beveridge et al., 1999; Garcia et al., 1999; Perez et al., 2009). The 2004 world-wide annual consumption of QACs was reported as 500,000 tons (CESIO, 2004) and was expected to reach or exceed 700,000 tons (Steichen, 2001).

This study compares standard toxicity tests with brief exposure and spill simulated exposure profiles for aniline and BAC on different marine species which are likely to be impacted by chemical spills.

MATERIAL AND METHODS

Test chemicals and analysis

Aniline (CAS number 612-008-00-7, ACS grade ≥99.5%), and benzalkonium chloride (CAS number 63449-41-2, ACS grade ≥95.0%) were purchased from Sigma-Aldrich UK. Solutions were prepared in filtered (0.2 µm) seawater and used to make appropriate stock solutions and test dilution series the same day of the test. Samples of the test dilution series were analysed to determine the concentrations of the chemicals in the test solutions to confirm actual concentration at the beginning of the test.

Analysis of concentrations in the test solutions were conducted by the Environment Agency National Laboratory Service (NLS). Samples of aniline were analysed by Gas Chromatography Mass Spectrometry (GCMS). This method has an assured detection limit of 2 µg l^{-1}. Samples of benzalkonium chloride (BAC) were analysed using Liquid

Chromatogram interfaced to a Triple Quadrupole Mass Spectrometer. The detection limit for benzalkonium chloride is 5 µg l^{-1}.

Test organisms and culture conditions

Organisms belonging to different groups were chosen for this study. The harpacticoid copepod *Tisbe battagliai* was chosen as a sensitive, ecologicaly relevant and frequently dominant secondary producer in marine zooplankton (Hart, 1990). Animals used in this study were obtained from Guernsey Sea Farms (Guernsey) and kept in the laboratory according to international accepted procedures (ISO 14669, 1999) with the exception of feeding which consisted of a mixture of concentrated algal feeds (Isochrysis 1800 and Tetraselmis 3600, Reed Mariculture, San Jose, CA, USA) at a 1:1 ratio. *Fucus vesiculosus* was chosen for this study as a representative brown macroalgae and thus at the base of the marine foodweb. This was collected at a site near to the Cefas Lowestoft laboratory (Suffolk, UK) the day before tests were conducted. *Ceramium tenuicorne* is a filamentous red macro algal species that can grow up to 10cm in length, it is widely distributed in temperate waters and is found in both brackish and marine waters (Ekelund, 2005). Cultures of *C. tenuicorne* were held at Cefas' Lowestoft laboratory in sterile seawater with added nutrients according to methods described by Ekelund, 2005 and ISO 10710:2010, at 22 ± 2°C, salinity of 20 ppt and a light:dark cycle of 16:8 hours at a light intensity of 35 ± 5 µmol m^{-2} s^{-1}. Salinity was increased by 3 ppt every other day to reach a salinity of 30 ± 1 for test conditions. Cultures were acclimatised for at least 1 week before testing in natural seawater. All the tests were carried out in natural seawater.

All toxicity tests were run using 0.2 µm filtered natural seawater. Tests using the crustacean *T. battagliai* and brown seaweed *F. vesiculosus* were carried out at 20 ± 2 °C while tests using the red seaweed *C. tenuicorne* were conducted at 22 ± 2 °C at the Cefas

laboratory in Lowestoft. Water qualities were recorded at the beginning and at the end each test and at each solution change.

Tests using T. battagliai

Standard toxicity tests as well as brief and spill simulation exposures were performed for *T. battagliai* with aniline and BAC. All the tests were performed according to ISO 14669 (1999) with some modifications for brief exposures or spill scenarios. Briefly, juvenile copepods (6 ± 2 days old) were used for all the toxicity tests. Nominal concentrations tested during standard toxicity tests were 0, 0.3, 1, 3, 10, 30 mg l^{-1} and 0, 0.3, 0.6, 1.2, 2.5, 5 mg l^{-1} for aniline and BAC respectively. Observations of mortality were made using a stereomicroscope at 24 and 48 hours. 12 well plates with 5 animals each well were used as test replicates. 4 replicates were used per treatment and 8 replicates in the control. For brief exposure tests with aniline, juveniles were exposed to 0, 10, 30, 100 and 300 mg l^{-1} for 1 and 2 hours. For BAC, juveniles were exposed to 1 and 5 mg l^{-1} for different time periods between 1 and 48 hours.

A parallel zinc reference test was carried out to ensure the batch of organisms tested were of similar sensitivity to previous batches.

Test using C. tenuicorne

All *C. tenuicorne* toxicity tests were carried out following the ISO 10710:2010 guideline with modifications. A 48 hour test was conducted on growing tips of *C. tenuicorne* for BAC as a range finder for further time based tests and as an indication of toxicity over a relatively short period. Growth was used as the measure of chemical effect. Briefly, growing tips of length between 0.6 and 1.2mm were cut from plants using a binocular microscope the day before the start of the test. Two tips were placed into 6 well polystyrene culture plates with 5ml of solution. For standard toxicity test, nominal test concentrations used for benzalkonium chloride exposure were 0, 0.05, 0.1, 0.5, 1 and 5 mg l^{-1}. Brief exposure tests

were performed exposing *C. tenuicorne* to 0.5 mg l^{-1} BAC for 0, 6, 12, 24 and 30 hours and to 5 mg l^{-1} 0, 1, 3 and 6 hours. Test conditions were 22 ± 2°C, light:dark cycle 14:10 hours and light intensity 70 ± 100 µmol m^{-2} s^{-1}.

At the end of the test *C. tenuicorne* tips were preserved in 0.4% buffered formalin and stored at 5 ± 3°C until the measurement of the samples. Tips length was then analysed at the stereomicroscope using Leica Application Suite Version 4.7.0 (Leica microsystems, UK Limited).

F. vesiculosus

96 hour tests were conducted on sporlings of *F. vesiculosus* for BAC. Germination success and frond growth were measured as sensitive and ecologically relevant end points to determine effect concentrations.

Approximately 100 receptacles of *F. vesiculosus* were used for each toxicity test. After collection, receptacles were washed in filtered seawater and kept damp overnight at 5 ± 3 °C. Receptacles were then placed into filtered seawater at 20°C in direct sunlight. Sporlings were concentrated in approximatively 50 ml filtered seawater using a 25 µm mesh. Sporlings were then inoculated to achieve 50 sporlings/ml directly on 6 well plates containing 5 ml of filtered natural seawater or test solution. A glass cover slip (22mm x 22mm) was added in each well to allow attachment of the sporlings. Coverslips with sporlings attached were moved into test wells containing freshly made up test solution at relevant intervals. 4 replicates were used for each treatment group and 8 for the control. Slides were moved to fresh solutions after 48 hours for BAC.

The nominal concentrations tested were 0, 0.05, 0.1, 0.5, 1 and 5 mg l^{-1} BAC. For brief exposure tests *F. vesiculosus* sporlings were exposed to 1 and 5 mg l^{-1} BAC for 0, 1, 3, 6, 20, 26 and 48 hours. Coverslips were preserved after 96 hours in 0.4% buffered formalin

solution and refrigerated at 5 ± 3 °C until they could be observed and measured. Coverslips with attached sporlings were observed using a stereo-microscope and camera while they were within well plates, in solution using Leica Application Suite Version 4.7.0 (Leica microsystems, UK Limited).

Spill profile exposures

Spill profiles used for toxicity tests were obtained from a representative location in the spill plume for the Poole Bay using the hydrodynamic transport model ChemMap (McCay et al., 2006). The profiles used for the simulations refer to possible spill situations with duration of four hours and based on an original total quantity spilled of 1000 t. However, a smaller spill volume of 60 -100 tonnes was investigated for BAC and so the time weighted average (TWA) values were scaled accordingly to 1.25 mg l^{-1} for *T. battagliai* juveniles and 0.8 mg l^{-1} for *F. vesiculosus* and *C. tenuicorne*. From the modelling a representative exposure profile was built up as a number of timed phases (see Table 1).

Statistical analysis

All statistical analyses for effect times and concentrations were performed in CETIS v1.8.0 (Tidepool Scientific, USA). When appropriate, survival and sub lethal effects were analysed using linear (Probit model) regression if possible. When this could not provide an appropriate fit to the data, linear interpolation or Trimmed Spearman-Karber method was used. Analyses were performed against verified concentrations.

The Bartlett Homogeneity of Variance test and the Shapiro–Wilks Normality test respectively within the CETIS package were used to confirm whether the dataset met the assumptions required for a normal distribution and as a consequence identified appropriate tests of significance between the response of treatment groups and the control. NOEC (no observed effect concentration) and LOEC (low observed effect concentration) values were calculated using one-way ANOVA. Comparison between groups was then obtained using Wilcoxon/Bonferroni Adjusted Test or Dunnet's multiple comparison test. For brief exposure studies where time-based as opposed to concentration-based assessments were made, NOEL refers to no observed effect level (hours) and LOEL to lowest observed effect level (hours). Both of these latter parameters were calculated using the same methods as for NOEC and LOEC. Spill simulation experiments and brief exposure experiments were analysed using GraphPad Prism 6 Version 6.04 (GraphPad Software, Inc.). Normality was tested using D'Agostino & Pearson omnibus normality test. Data were not normally distributed, so, non-parametric analysis of variance was performed (Kruskall-Wallies test). Dunn's multiple comparisons test was used to detect differences between groups.

Table 1. Duration of each test phase and concentration of chemical for the different phases of the spill simulations.

Phase	**Duration (hours)**	***T. battagliai* Aniline (mg l^{-1})**	***T. battagliai* BAC (mg l^{-1})**	***F. vesiculosus* *C. tenuicorne* BAC (mg l^{-1})**
1	8	34.5	2.5	1.6
2	3.25	0	0	0
3	2.75	31.5	2.25	1.4
4	11.75	11.5	0.8	0.5
5	3.75	3.5	0.25	0.15

RESULTS AND DISCUSSION

The issue of chemicals transported at sea was firstly tackled by a European funded project which identified 100 chemicals mostly transported between European ports (HASREP, 2005) then used by Neuparth et al. (2011) to identify a sub-list of 24 priority HNS for which there is a lack of marine ecotoxicology data. Aniline was chosen because because of its listing as a priority chemical identified by Neuparth et al. (2011) while BAC was selected as an example of a chemical with contrasting toxicological mode of action.

Table 2. Effect concentration values (mg l^{-1}), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) calculated using measured concentrations of BAC with *T. battagliai*, *C. tenuicorne* and *F. vesiculosus*.

BAC (mg l^{-1})	***T. battagliai*** **(48 hrs)**	***C. tenuicorne*** **(48 hrs)**	***F. vesiculosus*** **(48 hrs)**	***F. vesiculosus*** **(96 hrs)**
NOEC	0.29	0.07	0.38	<0.02
LOEC	0.54	0.42	1.11	0.02
EC_{50} (95% LCL -UCL)	0.81 (0.69-0.92)	0.78 (n/a-1.88)	0.70 (0.70-0.70)	0.30 (0.25-0.34)

Table 3. Lethal concentration values (mg l^{-1}), no observed effect concentration (NOEC) and low observed effect concentration (LOEC) calculated using measured concentrations of aniline with *T. battagliai*.

Aniline (mg l^{-1})	***T. battagliai*** **(48 hrs)**
NOEC	0.3
LOEC	1
LC_{50} (95% LCL -UCL)	1.96 (1.01 - 3.77)

Toxicological effect concentrations of aniline and BAC are shown in Table 2 and 3. BAC showed 48 hrs-EC_{50}s of similar values for the three tested species with lowest value for *F. vesiculosus* (0.70 mg l^{-1}) and highest value for *T. battagliai* (0.81 mg l^{-1}). Higher toxicity

was observed for a 96 hours exposure of *F. vesiculosus* (0.30 mg l^{-1}). Aniline toxicity was only evaluated for *T. battagliai* and showed a 48-hours EC_{50} of 1.96 (1.01-3.77) mg l^{-1}. All the values of effect concentrations reported in this section were calculated using measured concentrations of the chemical.

Spill profiles were modelled using CHEMMAP. The results of the profile investigations showed that the duration of elevated chemical concentrations for a spill in a position near the coast, with less opportunity for dispersion and dilution, can last up to 30 hours, while in a position offshore the presence of the chemical is generally brief (around 1 hour). The concentrations of a chemical in a spill situation near the coast can vary due to the effect of currents and tides, together with the amount spilled, with an alternation of phases of high concentrations followed by lower concentration phases. The behaviour and mode of action of a chemical can highly influence the toxicity and the impact of a spill. Some representative profiles were tested and some studies were conducted applying brief exposures that represented a potential worst case peak exposure during a spill. Exposure of *T. battagliai* to up to 300 mg l^{-1} aniline for 1 and 2 hours did not produce any mortality during the observation period (up to 168 hours). Data are not shown. Similar time exposure to BAC (3 hours) to a much lower concentration (5 mg l^{-1}) produced a mortality above 50% (Figure 1).

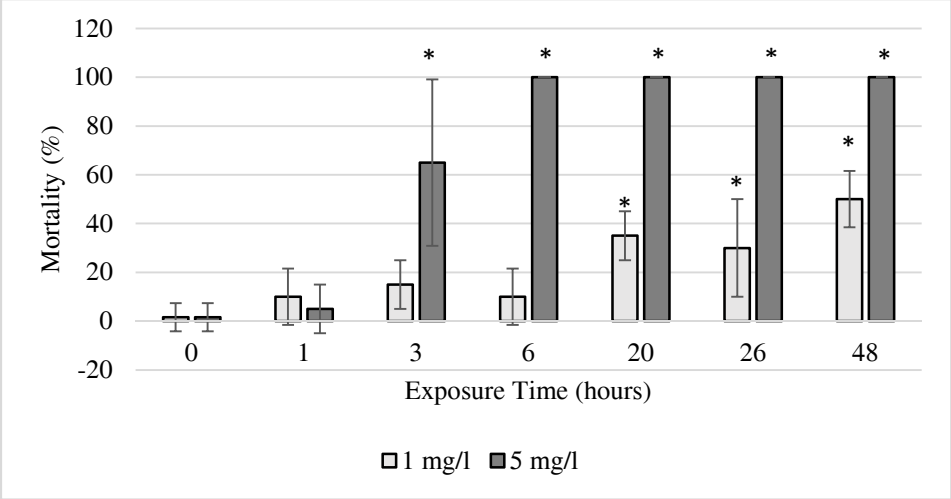

Figure 1. Percentage mortality of *T. battagliai* at 48 hours exposed to 1 and 5 mg l^{-1} for different times between 1 and 48 hours. * indicates significant differences to the control ($p<0.05$).

C. tenuicorne plants were exposed for 0 to 30 hours to 1 mg l^{-1} BAC and for 0 to 6 hours to 5 mg l^{-1} BAC and recovery allowed for 7 days. Total loss of pigments (bleaching) was observed after 1-hour exposure to 5 mg l^{-1} (data not shown). Plants exposed to 1 mg l^{-1} BAC showed a 40% reduction of growth after a 30 hours exposure and no recovery after 7 days (Figure 2). ET50 was calculated for groups exposed to 1 mg l^{-1} benzalkonium chloride and was >30 hours (data not shown).

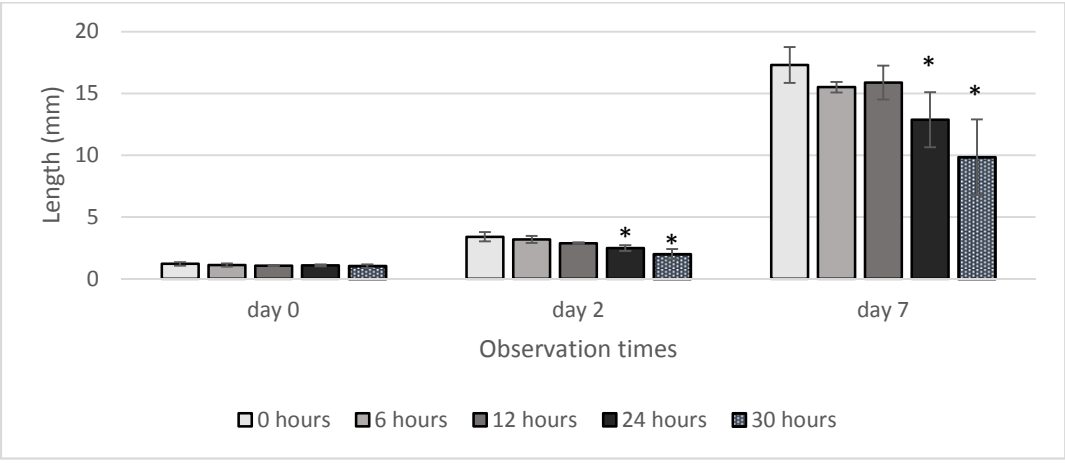

Figure 2. *C. tenuicorne* length (mm) measured at day 0, day 2 and day 7 for plants exposed to 1 mg l^{-1} BAC for different periods (0-30 hours) and allowed to recover until day 7.

F. vesiculosus sporlings were exposed to 1 mg l^{-1} BAC for different periods (between 3 and 26 hours) at two different developmental stages. Effect time calculations for the pre-germination and post-germination tests are shown in Table 4. Germination was used as a second endpoint and showed slightly lower sensitivity. The 1 mg l^{-1} BAC 50% effect time for this endpoint was 16 (13-20) hours while that calculated for the frond length was 15 (14-16) hours. The 50% effect time calculated on frond length (growth) in sporlings exposed post-germination was very similar to that calculated for the pre-germination test 13 (10-18) hours. Sporlings exposed to 5 mg l^{-1} BAC pre-germination showed 50 % reduction of growth after 1 hour exposure and no germination for exposures periods longer than that (data not shown).

Table 4. Effect Time (ET) calculated for *Fucus vesiculosus* exposed to 1 mg l^{-1} BAC for different periods of time between 0 and 26 hours in the pre-germination test and post-germination test. NOEL is the no observed effect time and LOEL is the low observed effect time.

Hours	Germination	Pre-germination Growth	Post-germination Growth
NOEL	6	3	<3
LOEL	20	6	3
ET_{50} (95% LCL-UCL)	16.62 (13.60-20.25)	14.8 (13.19-16.18)	13.12 (9.81-18.14)

In this study, spill simulation experiments were performed on the basis of a spill profile modelled for a position near Poole Bay of a duration of 30 hours. For experimental reasons, this profile was then divided in five phases which were scaled to obtain the relevant time weighed averages (Table 1).

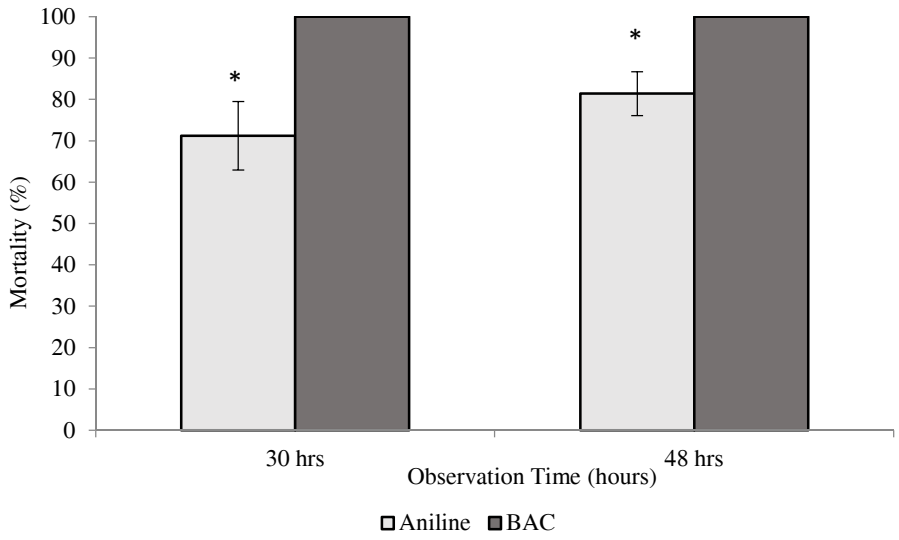

Figure 3. Percentage mortality of *T. battagliai* exposed to a 30 hours spill profile with aniline (TWA 17.39 mg l^{-1}) and BAC (TWA 1.25 mg l^{-1})

T. battagliai was exposed to spill simulations for both, aniline and BAC. For aniline, the simulation represented a spill of 1000 tonnes, a duration of 30 hours and a TWA over 48 hours of 17.39 mg l^{-1}. Mortality observed for this was >70% although the observed control mortality was >20%. For the same location a BAC spill was simulated representing a smaller volume spilled (100 tonnes) and a TWA of 1.25 mg l^{-1} produced 100% mortality.

Spill simulations with *C. tenuicorne* and *F. vesiculosus* were only performed for a scenario in which 60 tonnes of BAC are spilled representing a TWA of 0.8 mg l^{-1}. Based on the measured concentrations for the spill profile the calculated TWA was 0.69 mg l^{-1}. Results obtained for length of *C. tenuicorne* are shown in Figure 4 and *F. vesiculosus* in Figure 5. Significant growth reduction (>50%) was found at both species for groups exposed to spill profile and TWA. No significant differences were found between the two treatment groups. Percentage of germination was also observed for *F. vesiculosus* and was not affected by the exposure to the TWA (> 75%). Spill simulation treatment groups showed only 30% germination and this was significantly different from both control and TWA group ($p<0.05$).

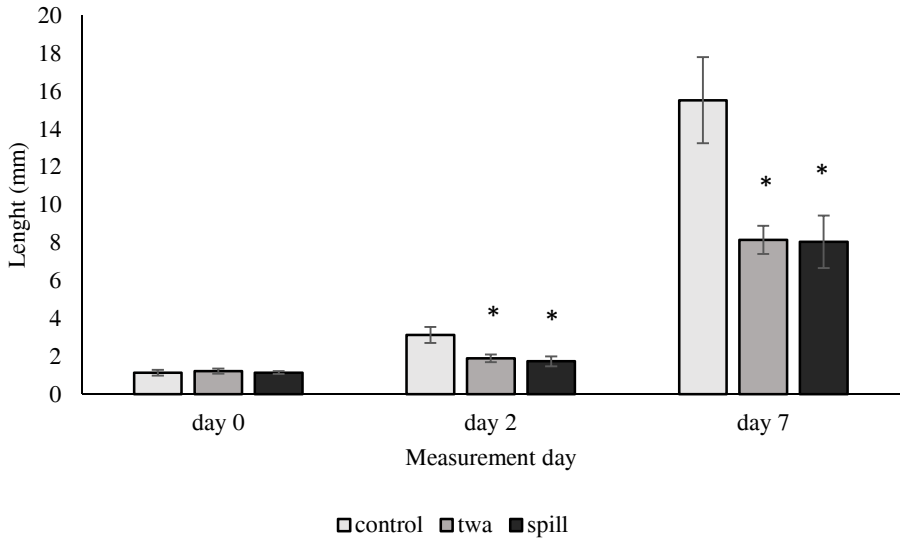

Figure 2. Length (mm) of *C. tenuicorne* obtained during the spill simulation experiment with BAC at day 0, day 2 and day 7 for control, spill and TWA. * indicate statistical differences with control ($p<0.05$).

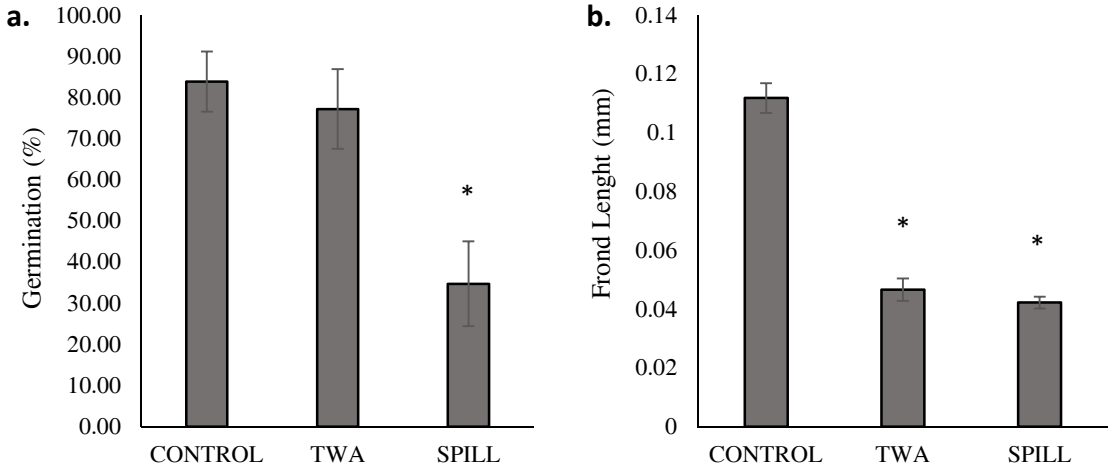

Figure 3. Germination (%) (a.) and frond length (mm) (b.) of *F. vesiculosus* obtained during the spill simulation experiment with BAC after 48 hours for control, spill and TWA. * indicate statistical differences with control ($p<0.05$).

Our results indicate that for particular spill situations close to shore, exposure profiles typically show multiple peaks but the peaks are frequently of only a few hours in duration. In the Poole bay spill scenario used in this study, a spill of 60 -100 tonnes of BAC, because of its potency following relatively brief exposures, is likely to cause significant impact on benthic species i.e. cause mortality in excess of 50% in a number of invertebrates and irreversible inhibition of growth of a number of plant species across an area of several square kilometres. A similar scale aniline spill would be unlikely to show any measurable effects yet the toxicity of both substances in constant exposure studies is of a similar order of magnitude.

In conclusion, for chemicals with specific modes of toxic action even short exposures to relatively higher concentrations may result in sufficient chemical uptake to cause irreversible toxic effects. Depending upon the threshold concentration at which such effects occur for a specific test organism, such effects may not be predicted in standard constant exposure studies. In those cases, standard toxicity tests alone are not sufficient to evaluate the impact of chemical spills. Time based studies (brief exposures) and possibly limited spill profile testing may better inform on the likely impacts for selected high priority substances but in addition physicochemical property data can also be used to predict the mode of toxicity and the likelihood of chemical spills causing significant impacts even in modest spills.

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