

MARINER



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Enhancing HNS preparedness
through training and exercising

Guidelines and protocols for environmental monitoring and impact assessment of Hazardous and Noxious Substances (HNS)

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¹Authors contribute equitably to the book.

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Introduction

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). Due to their diverse physico-chemical nature, HNS exhibit a wide range of behaviours in the environmental compartments and toxicities to marine organisms. Therefore, the level of knowledge available on the toxicity and risk associated with marine HNS spills is limited in comparison with other contaminants (Neuparth et al., 2011).

Presently, monitoring both the chemical and ecological status is recognized as essential towards the main aim of a “good water status” (Water Framework Directive 2000/60/EC, European Commission, 2000). Chemical assessment indicates the presence and/or concentration of a chemical in the various marine compartments. It is unlikely that fully validated, targeted analytical methods will be available for the wide range of possible spilled chemicals, as most are not included in routine monitoring programs. Nevertheless, the combination of liquid or gas chromatography–mass spectrometry (LC-MS and GC-MS, respectively) have the capability to be used for the analysis of most compounds. On the other hand, ecological monitoring is based on biological changes at the population/community level. Nevertheless, although relevant, changes in the structure of biological communities cannot be used as a preventive tool since they reflect the responses of the organisms once the alteration of the ecosystem has taken place (Martinez-Haro et al., 2015). In this context, biological effects methods, namely bioassays and biomarkers, are proposed to contribute to the smooth integration of the chemical and biological information, allowing a better understanding of cause-effect relationships (European Commission, 2009, 2010). More precisely, these tools allow to investigate the cause of failing ecological status and whether pollutants are responsible for it, closing the gap between chemistry and ecology (ICES, 2007). Ecotoxicological bioassays consist of the exposure of test organisms in controlled conditions to matrices (water, sediment) whose toxicity is intended to assess, either in the laboratory or under field conditions, and the measurement of ecologically-relevant quantitative responses (Martinez-Haro et al., 2015). In turn, biomarkers are defined as “quantitative measurements of changes occurring at cellular, biochemical, molecular, or physiological levels, that can be measured in cells, body fluids, tissues or organs within an organism and that may be indicative of xenobiotic exposure and/or effect” (Vidal-Liñán and Bellas, 2013). In this sense, both bioassays and biomarkers, are sensitive, cost-effective, early-warning systems of stressors that may potentially impact ecosystem function. The combination of the different approaches, at different levels of organization, can provide an integrated view of the environmental stresses affecting an ecosystem, in order to achieve the best evaluation of the ecosystem community health.

This book follows holistically the triad approach. The first axis involves HNS chemical assessment with a summary of several analytical techniques which can be used to analyse the chemical concentration of priority HNS, selected according their intrinsic

characteristics (i.e. physico-chemical and toxicological properties) and traffic ranking (Neuparth et al., 2011). Behaviour, physico-chemical and ecotoxicological data on these priority HNS was systematized (Cunha et al., 2016) and made available on an online database (<http://www.ciimar.up.pt/hns/substances.php>). The second axis involves an inventory of various types of bioassays and biomarkers, which includes whenever possible standardized tests issued by OECD (Organization for Economic Cooperation and Development), ASTM (American Society for Testing Materials), EPA (U.S. Environmental Protection Agency), ICES (International Council for the Exploitation of the sea), and non-standardized tests performed within research peer-reviewed articles. As a third axis, propositions for ecological guidelines are made. Efforts have been made to ensure the information on this book reflects the original sources. However, users should refer to the original publication to thoroughly comprehend the test. It is anticipated that the environmental risk assessment community, including scientific researchers, environmental agencies and regulatory bodies, as well as the competent authorities involved in HNS spill responses will utilize this book as an important tool to support environmental monitoring and impact assessment associated with HNS spills.

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Chapter 1. Chemical assessment

1.1. Analytical methods for the quantification of priority HNS in water matrices

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Contaminant analysis of seawater can be an element of integrated monitoring and assessment, where chemical and biological effects measurements are combined, in order to assess potential harm to living resources and marine life (OSPAR, 2012). The role of chemical measurements in integrated chemical and biological effects monitoring programmes is to help in the identification of the chemical causes responsible for the observed biological effects.

It should be noted that analyses at low concentrations, as those generally found in seawater samples, require extensive experience and should be made by expertise laboratories. Several analytical methods can be employed to analyse the same compound, provided that all performance criteria on the method is met. A minimum performance criteria consistent with Commission Directive 2009/90/EC, includes an uncertainty on measurements < 50 %, estimated at the level of the relevant Environmental Quality Standard, and a limit of quantification (LOQ) \leq 30 % of the Environmental Quality Standard. If no method meets the minimal performance criteria, the best available analytical method, not entailing excessive costs, should be used.

In this chapter, adequate analytical methods for the quantification of priority Hazardous and Noxious Substances (HNS) (Table 1) can be found. Whenever possible preference is given to protocols for seawater samples analysis.

Table 1 – List of priority HNS and their behaviour in seawater.

Priority HNS	Behaviour in seawater^a
Benzene	E
Cyclohexane	E
Heptane	E
Hexane	E
Toluene	E
1-Nonene	FE
Octane	FE
Styrene	FE
m-Xylene	FE
Butyl acrylate	FED
Aniline	FD
Cyclohexylbenzene	F
Pentylbenzene	F
Decanoic acid	Fp
Di (2-ethylhexyl) adipate	Fp
1-Dodecanol	Fp
Isononanol	Fp
1-Nonanol	Fp
4-Nonylphenol	Fp
Tetrachloroethylene	S
m-Cresol	SD
Nitrobenzene	SD
Trichloroethene	SD
Acrylonitrile	DE

^a D: dissolver; E: evaporator; F: floater; S: sinker; DE: dissolver/evaporator; FD: floater/dissolver; FE: floater/evaporator; FED: floater/evaporator/dissolver; Fp: persistent floater; SD: sinker/dissolver.

Searches were conducted in various information sources: a) Scopus (<http://www.scopus.com/>); b) ISI Web of Knowledge (WoK, <http://wokinfo.com/>) databases; c) Pubmed (<https://www.ncbi.nlm.nih.gov/pubmed/>), d) Google (<https://www.google.pt/>); e) documentation from the Agency for Toxic Substances and Disease Registry (ATSDR) and f) documentation from Environmental Protection Agency (EPA). Several keywords and combinations of search terms were used: “name of HNS of interest” in combination with one or more of the following terms “AND quantification”, “AND determination”, “AND concentration”, “AND water”, “AND seawater” and “AND EPA method”. Description of an analytical method in water matrices was not found for the following HNS: 1- nonene (CAS number 124-11-8, UN number 2057), pentylbenzene (CAS number 538-68-1, UN number 3082), decanoic

acid (CAS number 334-48-5), isononanol (CAS number 27458-94-2, UN number 1993) and 1-nonanol (CAS number 143-08-8, UN number 3082).

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1.1.1. Benzene

Principle of the test

Benzene is a natural constituent of crude oil and is one of the elementary petrochemicals. Though it is formed organically, the vast majority of the benzene in the environment is of anthropogenic source. Benzene is primarily used as a precursor to the manufacture of chemicals with more complex structure, such as ethylbenzene, cumene and cyclohexane, which are massively produced. As benzene is a human carcinogen, most non-industrial applications have been limited. In animals, exposure to food or water contaminated with benzene can damage the blood and the immune system and can cause cancer.

Headspace solid-phase microextraction (HS-SPME) and quantification by gas chromatography with flame ionization detection (GC-FID) is applied to seawater samples to extract and measure benzene (CAS number 71-43-2, UN number 1114) concentrations levels ($\mu\text{g/L}$).

Analytical procedure

Preparation of standard stock solutions

1. Benzene must be of analytical grade and methanol of HPLC-grade. Purified water should be obtained from a Milli-Q ultra-pure water system. The stock solution of benzene is prepared by adding 100 mg of the chemical into a 10 mL volumetric flask, diluting with methanol. A further dilution, by a factor of 10000 with methanol, is performed to obtain a working standard of 1 mg/L which is used to prepare spiked water solutions. All solutions are stored at 4 °C in a refrigerator.

Analytical method

2. The HS-SPME procedure is performed by transferring a water sample of 10 mL into a 15 mL glass vial, containing a magnetic stirring bar, with PTFE-coated septa. The vial is positioned in a water-jacketed vessel on a magnetic stirrer (1000 rpm) and kept at 25 °C with a circulating water bath for 20 min. A fiber coated with single walled carbon nanotubes (SWCNTs), annealed at 500 °C for 30 min in a H₂ stream, is exposed in the headspace of the sample about 1 cm below the septa, to extract benzene. The fiber should have a coating thickness about 80 µm and a specific surface of 380 m²/g.

3. After extraction, the fiber is immediately inserted into the GC injection port for thermal desorption at 300 °C for 5 min. The carryover is then measured with one blank injection.

4. The GC measurements are performed on a gas chromatograph equipped with FID and a split-splitless injection port. The separation of benzene is carried out on a DB-624 column (75m × 0.53mm, 3 µm in film thickness). Data acquisition and processing is done using star chromatography workstation. The GC oven temperature program is as follows: 50 °C held for 3 min, rate at 5 °C/min to 130 °C and held for 2 min. The carrier gas is high purity nitrogen with a pressure of 20 psi in the injection port. The injection port and detector temperatures are set at 300 and 250 °C, respectively. The pressure of the H₂ and air for the detector is 20 and 40 psi, respectively. Splitless mode is adopted.

Analytical performance

The linear range, correlation coefficient (r), limits of detection (LODs), limits of quantification (LOQs), and repeatability the method for the analysis of benzene in spiked water are listed in Table 2.

Table 2. Characteristic data for the described HS-SPME-GC method for the determination of benzene^a.

Compound	Linear range (µg/L)	r	LOD (µg/L)	LOQ (µg/L)	RSD (%)
Benzene	0.5 - 50.0	0.999	0.026	0.088	5.6

^a The analytical curve is constructed with five concentration levels of benzene. The concentration of the standard solution for the test of reproducibility and repeatability is 20 µg/L.

For more detailed information on the analytical method and parameters calculation consult Li et al., 2010.

More information

Benzene can be quantified in water samples by EPA method 524.2. “Measurement of purgeable organic compounds in water by capillary column gas chromatography-mass spectrometry”, with previous extraction by purge and trap procedures.

References

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1.1.2. Simultaneous measurement of cyclohexane, toluene and m-xylene

Principle of the test

Cyclohexane is used to make nylon, as a solvent, paint remover, and to make other chemicals. Toluene is also used as a solvent and thinner in dyes, and as a feedstock in chemical synthesis while m-xylene is used in the manufacture of coatings and plastics. Toluene and m-xylene are also abundant in petroleum products, such as crude oil. At normal environmental concentrations, and even to higher concentrations over a short period of time, is unlikely that these chemicals damage health significantly. However long-term exposure to higher concentrations are toxic. Similarly, at lower concentrations these compounds are unlikely to damage the environment, but at higher concentrations resulting from a spillage they can be moderately toxic to aquatic life.

Purge-and-trap combined with high-resolution gas chromatography and detection by mass spectrometry (GC-MS) can be used to analyse different organic volatile compounds. The method here described can be applied to seawater samples to determine cyclohexane (CAS number 110-82-7, UN number 1145), toluene (CAS number 108-88-3, UN number 1294) and m-xylene (CAS number 108-38-3, UN number 1307) concentrations in the ng/L range.

Analytical procedure

Preparation of standard stock solutions

1. Analytical stock solutions are prepared by two serial dilution steps in methanol. Standards are prepared by volume and calculated to mass by density. The stock solutions should be kept at -20 °C in a solvent free-compartment. Because of the high volatility of the analytes new standards must be prepared at the start of each new batch of samples. Stock solutions should be renewed after 2 weeks of use.

Preparation of blanks and reference materials

2. Blank water is prepared by purging 60 ml of natural seawater for at least 1 h at 45 °C and a He flow-rate of 50 mL min⁻¹. System blanks, laboratory reference materials (LRMs) and calibration materials (CMs) are prepared by injecting 5 mL of the appropriate analytical stock solution in water, purged to blank.

Sample preparation

3. Seawater sampling can be performed with a 10 L Niskin bottle equipped with a CTD-probe for continuous monitoring of temperature, salinity and depth. The samples should be immediately transferred to dark green glass bottles (volume 780 mL), by means of a silicone tube, which must be filled completely to avoid any headspace. Microbial degradation is prevented by adding HCl/H₂O (1/1; v/v) to obtain a final pH lower than 2. Finally, 5 mL of a deuterated surrogate solution in methanol, containing 50 µl of chloroform-d₁, toluene-d₈ and chlorobenzene-d₅, is injected. The recipients are sealed with PTFE tape and stored at 4 °C.

Analytical method

4. A sample aliquot of 60 ml is brought into an off-line purge-and-trap device, kept in a water bath set at 45 °C. 5 µL of the I.S. solution is added with a 10 µL Hamilton precision syringe. The sample is allowed to thermally equilibrate for 7 min before purging for 20 min at a rate of 50 ml He min⁻¹. The compounds of interest are trapped at room temperature on a custom made multi-bed sorbent trap, which consists of Tenax TA, Carboxen 1000 and Carboxen 1001. A 'water' trap, kept at -15 °C, is inserted between the purge vessel and the multi-bed sorbent trap.

5. The trap is then thermally desorbed at 275 °C for 15 min in an on-line CDS Peakmaster system. The analytes are focused in a cryotrap at -150 °C prior to injection into a GC-MS apparatus (e.g. Carlo Erba QMD 1000). Separation is done on a Rtx-502.2 fused silica capillary column (60 m × 0.32 mm I.D., 1.8 µm film). m-Xylene and p-xylene cannot be separated and are reported as a single value. Temperature programming of the GC and data acquisition are started simultaneously. The temperature of the GC-oven is held at 40 °C for 10 min, and then linearly increased to 150 °C at a rate of 10 °C min⁻¹, and finally to 220 °C at 8 °C min⁻¹. Temperature is kept at 220 °C for 10 min. The MS is operated in the selected ion monitoring mode. The ion source temperature of the mass spectrometer is held at 200 °C. The emission electron energy is set to 70 eV and the trap current to 150 µA.

Table 3. Selected ion masses and time windows for the mass spectrometer operating in the SIM mode, and internal standard (I.S.) used for quantification.

Compound	Selected ion masses (m/z)	Time window (min)	I.S.
Chloroform-d ₁	84, 86	14.00-15.45	I.S. ₁
Cyclohexane	41, 56, 84	15.45-16.15	I.S. ₁
a,a,a-Trifluorotoluene (I.S. ₁)	127, 145, 146	17.50-19.20	n.a.
Toluene-d ₈	70, 98, 100	19.70-21.20	I.S. ₁
Toluene	65, 91, 92	19.70-21.20	I.S. ₁
Chlorobenzene-d ₅	82, 117, 119	23.00-25.50	I.S. ₂
m/p-Xylene	91, 105, 106	23.00-25.50	I.S. ₂
p-Bromofluorobenzene (I.S. ₂)	95, 174, 176	25.50-27.00	n.a.

na= Not applicable.

Analytical performance

1. The limits of detection (LODs), precision and accuracy of the above described method are listed in Table 4.

Table 4. Limits of detection (LODs), precision and accuracy.

Compound	LOD (ng/L)	Precision (%)	Accuracy (%)
Chloroform-d ₁	0.80	4.4	99.8
Cyclohexane	1.25	9.7	100.3
Toluene-d ₈	0.24	7.2	102.4
Toluene	4.99	9.2	89
Chlorobenzene-d ₅	0.24	4.7	99.5
m/p-Xylene	2.83	5.9	93.5

For more detailed information on the analytical method and calculation of LOD, precision and accuracy consult Huybrechts et al., 2000.

2. Possible losses of target analytes can be monitored by determining the amount of deuterated surrogates recovered after analysis.

3. Analytical quality control charts (AQCCs) can be constructed for each analytes by replicate at random analyses of a laboratory reference material (LRM) over a well-defined time-interval. The results are used to establish warning limits (WLs) and control limits (CLs). An LRM can be analysed in duplicate prior to each batch of samples and results check to fall within CLs for the analytes.

More information

Toluene and m-xylene can be quantified in water samples by EPA method 524.2. "Measurement of purgeable organic compounds in water by capillary column gas chromatography-mass spectrometry", with previous extraction by purge and trap procedures. Cyclohexane can be quantified in water samples by EPA method 1666.

Compound concentrations are determined by gas chromatography-mass spectrometry (GC-MS) using isotope dilution technique, with previous extraction by purge and trap procedures.

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1.1.3. Simultaneous analysis of heptane and octane

Principle of the test

Heptane is a test fuel component, which is also widely applied in laboratories as a non-polar solvent. Octane is an important constituent of gasoline. Both heptane and octane can cause eye and skin irritation, and central nervous system depression. Furthermore, they are very toxic to aquatic life.

Heptane (CAS number 142-82-5) and Octane (CAS number 111-65-9) of the water-soluble fraction (WSF) of gasoline in seawater can be analysed by gas chromatography-mass spectrometry (GC/MS), with previous purge-and-trap procedures at µg/L levels.

Analytical procedure

Analytical method

1. The preparation of a concentrated extract for GC/MS analysis is performed by taking about 1.5 L of the WSF in a separatory funnel, extracting three times with 10 mL portions of 3:7 mixture of hexane:dichloromethane. The combined extracts are dried over anhydrous sodium sulfate, filtered, and concentrated on a rotary evaporator to about 5 mL and then under a stream of nitrogen to dryness. The residue is taken up in 500 µL of methylene chloride.

2. The volatile compounds in the WSF are analysed by purge-and-trap chromatographic analysis with a flame ionization detector. The GC is linked to purge-and-trap

concentrator. A cryofocusing module is employed to refocus the volatile desorbed from the trap. Data is acquired and reprocessed by chromatography Software. A 3-mL sample of the WSF is introduced into the purging vessel with a Luer-lock syringe. For purging and trapping n-heptane and n-octane the sample is purged for 11 min with helium. The purged volatile compounds are trapped on a Tenax trap. The trap is heated to 225 °C to desorb and kept at the same temperature for 2 min during desorption. The desorbed compounds are refocused at the head of an analytical column at -150 °C after which the capillary interface is heated to 180 °C and the compounds introduced into the GC.

3. The conditions for GC analysis are as follows: fused silica capillary column, coated with SPB-624, 1.40 µm film thickness, 30 m long, 0.25 mm internal diameter. Helium is used as the carrier gas at 2 mL/min. Initial column temperature is 40 °C, maintained for 5 min and then programmed to 220 °C at 4°/min. The final temperature is held for 10 min. A flame ionization detector is used for detection. An external standard method is employed for quantitation.

4. The identities of the peaks are ascertained by linking purge-and-trap GC with MS. The GC is coupled with a quadruple mass spectrometer. The conditions used for GC analysis are the same as given for purge-and-trap/GC. The mass spectra of unknown peaks are compared with those standards as well as with the EPA/NIH mass spectral database.

Analytical performance

The composition in n-heptane and n-octane of the WSF prepared at two different temperatures is presented in Table 5.

Table 5. Composition in n-heptane and n-octane of WSF of gasoline (90-octane leaded) in seawater (µg/L).

Compound	a	15 °C			25 °C		
		2 h	4 h	24 h	2 h	4 h	24 h
n-Heptane		9	16	65	22	12	70
n-Octane		10	10	11	7	5	32

^a Stirring hours of gasoline with seawater.

For more detailed information on the analytical method consult Saeed and Al-Mutairi, 1999.

More information

n-Heptane can be quantified in water samples by EPA method 1666. Compound concentrations are determined by gas chromatography-mass spectrometry (GC-MS) using isotope dilution technique, with previous extraction by purge and trap procedures.

References

U.S. EPA Method 1666, Revision A for “Volatile Organic Compounds Specific to the pharmaceutical manufacturing industry by isotope dilution GC/MS”. U.S. Environmental Protection Agency, 1998.

Saeed, T., Al-Mutairi, M., 1999. Chemical composition of the water-soluble fraction of the leaded gasolines in seawater. *Environ Int.* 25(1): 117-129.

1.1.4. Hexane

Principle of the test

Hexane is a significant constituent of gasoline. Hexane is widely used as cheap, relatively safe, largely unreactive, and easily evaporated non-polar solvent. To humans this compound is neurotoxic, and is often used as a narcotic as well. Acute exposure to hexane leads to numbness in the extremities, dizziness, confusion, nausea, headache, and irritation of the eyes, nose, throat, and skin. Repeated exposure to hexane may have adverse health effects over the long term including decreased motor nerve conduction, changes in vision, and muscle weakness. The substance is toxic to aquatic organisms.

Hexane (CAS number 110-54-3, UN number 1208) can be quantified in water samples by EPA method 1666. Compound concentrations are determined by gas chromatography-mass spectrometry (GC-MS) using isotope dilution technique, with previous extraction by purge and trap procedures.

Analytical procedure

Analytical method

1. Briefly, stable, isotopically labelled analogues of hexane are added to the sample and the sample is purged with an inert gas at 45 °C in a chamber designed for water samples, as appropriate. In the purging process, the volatile compounds are transferred from the aqueous phase into the vapour phase, where they are passed into a sorbent column and trapped. After purging is completed, the trap is back flushed and heated rapidly to desorb the compounds into a gas chromatograph (GC). The compounds are separated by the GC and detected by a mass spectrometer (MS).
2. Identification of hexane (qualitative analysis) is performed by calibrating the GC/MS with authentic standards and storing a mass spectrum and retention time for each compound in a user-created library. A compound is identified when its retention time and mass spectrum agree with the library retention time and spectrum.
3. Quantitative analysis is performed by using extracted-ion current profile (EICP) areas. The GC/MS system is calibrated and the compound concentration is determined using an isotope dilution technique.
4. The quality of the analysis is assured through reproducible calibration of the GC/MS system.

References

U.S. EPA Method 1666, Revision A for “Volatile Organic Compounds Specific to the pharmaceutical manufacturing industry by isotope dilution GC/MS”. U.S. Environmental Protection Agency, 1998.

1.1.5. Styrene

Principle of the test

Styrene is employed in the manufacture of polystyrene, an important plastic, which is widely used as a packing material for food and consumer products and has been known to release styrene oligomers. Acute (short-term) exposure to styrene in humans' results in mucous membrane and eye irritation, and gastrointestinal effects while chronic (long-term) exposure results in effects on the central nervous system (CNS), such as headache, fatigue, weakness, and depression, CSN dysfunction, hearing loss, and peripheral neuropathy. Styrene is also anticipated to be a human carcinogen. At levels normally found in the environment it is unlikely that styrene causes significant harm to wildlife. However, exposure to high levels may have moderately toxic effects, particularly aquatic organisms. However, at levels normally found in the environment it is unlikely to cause significant harm.

Gas chromatography and detection by mass spectrometry (GC-MS) is applied to seawater samples to determine styrene (CAS number 100-42-5, UN number 2055) concentrations at $\mu\text{g/L}$ levels.

Analytical procedure

Preparation of standard stock solutions

1. Standard preparation of styrene monomer is as follows. Briefly, commercial polystyrene virgin pellets (average number of molecular weight: 500,000) are dissolved in 500 mL benzene, which are reprecipitated two times in 1500 mL methanol and allowed to dry for 10 days in vacuo (3 mm Torr) at 25 °C. Reprecipitated polystyrene chips are cut into small pieces with scissors, and these chips and heating medium (polyethylene glycol: PEG, reagent grade) are added into a round-bottomed flask which is submerged in a silicone oil bath, allowing for the chips to be thermally decomposed at 280 °C for 120 min. After thermal decomposition, the polystyrene itself and its unknown products are dissolved and recovered in 10 mL benzene. This mixture is transferred into a separatory funnel and was washed three times with 10 mL pure water to remove PEG. Then, the washed mixture is added to 10 mL methanol, and the portion of polymer is frozen at -80 °C to facilitate the precipitation, and then thawed and centrifuged at 5000 rpm for 10 min. The supernatant is mixed with approximately 10 g of anhydrous sodium sulfate and is allowed to stand overnight. After filtering, working standard material is prepared. These materials are determined quantitatively by gas chromatograph/mass spectrometer (GC/MS), adopting an internal standard method. Finally, the purity is confirmed after boiling point fractionation.

2. Dichloromethane (DCM) as an extraction solvent, biphenyl (BP) as a surrogate, and phenanthrene (PH) as an internal standard are reagent grade. Other chemicals are ACS grade reagents. All the solutions are made with high purity water from a Millipore ultrapurification system (> 18 MΩ cm).

Sample preparation

3. The seawater samples are taken at a water depth of 40 cm and subjected to cotton plug filtration using a stainless steel beaker. The volume of water sampled is 2.5 L, which is then extracted with DCM and with BP as the surrogate standard. The water samples in the field are immediately extracted using a portable shaker. The extraction conditions are 40 strokes min⁻¹ for 10 min and each sample is extracted four times with a total of 100 mL DCM.

4. In the laboratory, about 100 mL of DCM extract in the field is mixed with approximately 10 g of anhydrous sodium sulfate, and allowed to stand overnight. The extract is evaporated to dryness using a rotary evaporator at 30 °C. After the addition of 0.5 ppm PH as an internal standard, the eluate is completely dissolved in 1 mL of benzene. If the concentration is relatively high, this extract can be diluted to 10 mL or 100 mL benzene.

Analytical method

5. An GC with a quadrupole mass analyser is equipped with a 30 m × 0.32 mm i.d. (0.25 μm film thickness) DB-1 capillary column. The MS is operated at 70 eV in the electron ionization mode. A mass scanning range for styrene oligomers is from m/z 78 to 312. The ion source temperature is maintained at 200 °C, and interface temperature is 250 °C. The injector is kept at 250 °C, and an extract of 1 μL is injected in splitless mode. Helium is used as a carrier gas, with a constant flow of 1.4 mL min⁻¹. The following oven temperature is used: standby at 40 °C for 5 min, ramp to 290 °C at 15 °C min⁻¹, and then hold for 5 min. The total run time is 30 min. As referred above, BP and PH are used as surrogate and internal standards, respectively.

Analytical performance

1. To investigate the linearity of the response, a standard solution is injected into the GC/MS. The correlation coefficients of the calibration curves, the precision of the analysis, represented as relative standard deviation (RSD), the limit of detection (LOD) and limit of quantification (LOQ) of the method are presented in Table 6.

Table 6. Correlation coefficients, precision, limits of detection (LODs) and quantification (LOQ).

Compound	r ¹	RSD ¹ (%)	LOD (μg/L)	LOQ (μg/L)
Styrene	0.9966 - 0.9999	~ 10	3.3	10

¹ Standard solution in the range of 10-3000 μg/L.

2. The recovery of the spiked styrene on the seawater (n = 5) was 90.3 %.

3. Data quality assurance and quality control include laboratory and field blank samples consisting of deionized water in order to monitor changes in the sample preparation and the sensitivity of the instrument.

For more detailed information on the analytical method and calculation of method performance consult Saido et al., 2014.

More information

Styrene can be quantified in water samples by EPA method 524.2. "Measurement of purgeable organic compounds in water by capillary column gas chromatography-mass spectrometry", with previous extraction by purge and trap procedures.

References

Eichelberger, J.W., Munch, J.W., Bellar, T.A., "Measurement of Purgeable Organic Compounds in Water by Capillary Column Gas Chromatography/Mass Spectrometry Revision 4.1, Method 524.2". National Exposure Research Laboratory Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati OH 45268, 1992.

Institute for Health and Consumer Protection. European Chemicals Bureau. Existing Substances. 1st Priority List. Volume: 27. Styrene Part I - Environment. European Union Risk Assessment Report, 2002, United Kingdom.

Saido, K., Koizumi, K., Sato, H., Ogawa, N., Kwon, B.G., Chung, S-Y., Kusui, T., Nishimura, M., Kodera, Y., 2014. New analytical method for the determination of styrene oligomers formed from polystyrene decomposition and its application at the coastlines of the North-West Pacific Ocean. *Sci Total Environ.* 473-474: 490-495.

1.1.6. Butyl acrylate

Principle of the test

Acrylic polymers have broad applications in the manufacture of paints and paper coatings, emulsion adhesives, plastics, lighting, glass replacements, personal care products and even dental fillings. Butyl acrylate adversely affect human health as an eye and skin irritant. Butyl acrylate is moderately toxic to aquatic organisms.

Headspace gas-chromatography (GC) analysis with flame-ionization detection (FID) is applied to water samples to determine butyl acrylate (CAS number 141-32-2, UN number 2348) concentrations at mg/mL levels.

Analytical procedure

Analytical method

1. Headspace analysis is performed in the mode of single gas extraction. Anhydrous sodium sulfate (30 g) is placed in a standard 250-mL KZ medicine vial, and 150 mL of test water and 1 mL of orthophosphoric acid are added. The selected volume ratio of the gas and liquid phases (r) provides the representability of the sample necessary for analysis. The vial is closed with a rubber stopper, whose surface is protected with a Teflon film, and sealed with a metal clamp. After the dissolution of sodium sulfate, the vial is thermostated in a water bath heated to 75 °C for 1.5 h, and 5 mL of the vapour phase is taken with a heated medical syringe (to avoid vapour condensation) and injected into the injection port of the chromatograph.
2. All the analyses are on a chromatograph with a flame-ionization detector. A 100 × 0.3 cm steel column is filled with Chromaton N-AWHMDS (0.2–0.25 mm), to which 15 mass % of the stationary phase (polyethylene glycol adipate) is applied. The optimal conditions of chromatography are as follows: the temperatures of the column thermostat, injection port, and detector are 75, 190, and 170 °C, respectively; the flow rate of the carrier gas (nitrogen) is 30 mL/min; and the temperature of the water bath for attaining phase equilibrium is 75 °C. Peak areas measured in chromatograms are converted to the electrometer scale 50×10^{-12} A.
3. Quantitative determination is performed by the absolute calibration method using a series of aqueous calibration solutions containing butyl acrylate in the concentration range of 0.5 - 20 MPC (maximum permissible concentration, in potable water 0.01 mg/L). Calibration solutions are prepared from weighed portions of chemically butyl acrylate by the successive dilution method under conditions precluding losses of this volatile compound.

Analytical performance

1. The detection limit was 0.01 mg/L.
2. The relative errors for the concentration ranges of 0.5 - 5 MPC, 5- 10 MPC, and 10 - 20 MPC are 15, 5, and 1 %, respectively.

For more detailed information on the analytical method and calculation of method performance consult Baksanova et al., 2003.

References

Baksanova, L.E., Val'tsifer, V.A., Budnikov, V.I., Ryabchevskikh, Y.V., Kondrashova, N.B., 2003. Gas-chromatographic determination of butanol and butyl acrylate in aqueous solutions with the use of headspace analysis. *Journal of Analytical Chemistry* 58(1): 78-81.

1.1.7. Aniline

Principle of the test

Aniline is mainly used in the manufacture of precursors to polyurethane and other industrial chemicals. It has also been used for dyes, mordant agents, rubber chemicals, pharmaceuticals, fungicides, paints, etc. Aromatic amines, including aniline and other substituted derivatives, which are suspected to induce some tumours in humans, are well-known pollutants in environmental water.

The proposed analytical method involving liquid-liquid extraction and capillary gas chromatography-mass spectrometry determination can be used in aniline (CAS number 62-53-3, UN number 1547) analysis in environmental water samples at pg/mL or ng/mL levels.

Analytical procedure

Chemicals

1. Authentic standards of aniline (C₆H₅NH₂); dichloromethane (CH₂Cl₂), anhydrous sodium sulfate (Na₂SO₄), and sodium chloride (NaCl) of pesticide grade; and other high-grade reagents must be used. Aniline-d₅ and naphthalene-d₈ are used as surrogates or internal standards.

Analytical method

2. Liquid-liquid extraction: a water sample with a volume of 1000 mL is placed into a separatory funnel with 30 g NaCl, 100 mL CH₂Cl₂, and the surrogate standard (0.2 ppm aniline-d₅ in CH₂Cl₂, 0.5 mL). The sample is extracted twice with CH₂Cl₂ (100 mL and 50 mL).

3. Back extraction: the organic phases are combined and extracted with two separate 10 mL portions of 6N HCl.

4. Make alkaline: the 6N HCl solutions are combined, and 22 mL 6N NaOH is added slowly while the mixture is cooled in an ice bath.

5. Re-extraction: the aqueous solution is extracted with two separate 10 mL portions of CH₂Cl₂.

6. Dehydration: the organic phases are combined and dehydrated using anhydrous Na₂SO₄.

7. Concentration: the dichloromethane phase is concentrated to 3 - 5 mL by a Kuderna-Danish (KD) evaporative concentrator at atmospheric pressure. The solution is further evaporated to a volume of 1.0 mL under a nitrogen stream.

8. An aliquot is analysed by GC-MS-SIM.

9. A GC apparatus with a data processing system is employed. The analytical column used is a Carbowax 20M (25 m × 0.32-mm i.d., 0.3-µm film thickness). The GC temperature is held at 50 °C for 3min, and then increased at 5 °C/min to 185 °C. The temperatures of the injector, transfer line, and ion source are 250 °C, 250 °C, and 270°C, respectively. The carrier gas is helium at 2.0 mL/min (7.5 psi).

10. The mass spectrometer is operated at 70 eV and 300 µA in the electron-impact mode of SIM. The following ions are monitored: aniline (*m/z* 93) and aniline-d₅ (*m/z* 98).

Analytical performance

1. The limits of detection (LODs) and analytical precision of the above described method in a pure water sample (1000 mL) are listed in Table 7.

Table 7. Limits of detection (LODs), concentration and relative standard deviation (RSD).

Compound	LOD (ng/mL)	Concentration (ng/mL)	RSD (%)
Aniline	0.0042	0.03	2.3
		0.05	1.1
		0.1	1.4

2. Analyte recovery from environmental water samples (1000 mL) spiked with 100 ng of aniline is presented in table 8.

Table 8. Recovery of aniline from environmental samples.

Compound	Samples	Recovery (%)	RSD (%)
Aniline	Sea water	104 (75)	3.0 (6.2)
	River water	101 (68)	2.9 (4.2)

The values in parenthesis indicate the determined values calibrated with naphthalene-d₈ internal standard. Samples were spiked just prior to the GC-MS measurement.

For more detailed information on the analytical method and method performance calculation consult Okumura et al., 1996.

More information

Aniline can be quantified in water samples by gas chromatography-mass spectrometry (GC-MS) EPA method 8270D.

References

Okumura, T., Imamura, K., Nishikawa, Y., 1996. Determination of anilines in river water, sediment and fish samples by gas chromatography-mass spectrometry. *J Chromatogr Sci.* 34: 190-198.

Furukawa, K., Hasimoto, M., Kaneco, S., 2017. Optimization of analytical conditions for a rapid determination of aniline in environmental water by liquid chromatography/tandem mass spectrometry. *Anal Sci.* 33: 1189-1191.

U.S. EPA Method 8270D, "Semivolatile Organic Compounds by Gas Chromatography/Mass Spectrometry" by SW-846. U.S. Environmental Protection Agency, 2014.

1.1.8. Cyclohexylbenzene

Principle of the test

Cyclohexylbenzene is a solvent and penetrant that can be applied to industries including plastics, painting and adhesives. This compound can also be used for preparing two common intermediates for chemical products – phenol and cyclohexanone. To humans, cyclohexylbenzene may be fatal if swallowed and enters airways. Cyclohexylbenzene is also classified as a marine pollutant according to the International Maritime Dangerous Goods Code (IMDG). Solid phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS) is applied to drinking water samples for the determination of cyclohexylbenzene (CAS number 827-52-1, UN number 3295) concentrations in the µg/L range. Method validation is required for seawater samples.

Analytical procedure

Preparation of standard stock solutions

1. The reference pollutant compound, cyclohexylbenzene must be ultrapure grade and methanol of HPLC grade. Stock solutions are prepared in methanol containing 0.5-2.5 g/L of cyclohexylbenzene. Standard solutions containing 0.5 µg/L to 1 mg/L are prepared in doubly distilled water and mixed by magnetic stirring.

Analytical method

2. Extractions (SPME) are carried out on 10 mL aliquots of water samples and aqueous standards containing cyclohexylbenzene at different concentrations. The SPME fibres are completely immersed in the solutions with magnetic stirring at 400 rpm. Dioctyl phthalate (50 µL of a 33.6 mg/L solution) is added as internal standard. A medium polarity polydimethylsiloxane/divinylbenzene (PDMS/DVB) 65 µm fibre is used with the following optimum extraction conditions: an immersion time of 30 min at 60 °C.

3. The chromatographic and extraction conditions used for the analyses are as follows. Gas chromatograph with flame ionisation detection (FID). Column: Optima 17 (15 m × 0.53 mm I.D., 1 µm). Flow rates: nitrogen 10 mL/min, hydrogen 30 mL/min, air 300 mL/min. GC oven temperature program: 50 °C (hold for 1 min), increasing 10 °C/min to 270 °C (hold for 10 min). Detection temperature: 300 °C. Injector: splitless (3 min SPME extracts) temperature 270 °C. In applying the method to real samples, the chromatograph is coupled to a quadrupole MS detector at 280 °C with helium at a flow rate of 1 mL/min as carrier gas. All other operating conditions are as described for the standard solutions.

4. The concentration of reference cyclohexylbenzene is determined from the peak area. The response factor (concentration/peak area) is determined by directly injecting 2 µl of a 10 mg/l standard solution.

Analytical performance

A series of standards (spiked water samples) containing cyclohexylbenzene at concentrations ranging from 0.5 to 500 µg/L are prepared in purified water. The solutions are extracted by SPME according to the protocol described above and injected into the gas chromatograph. The repeatability expressed in terms of the coefficient of variation is determined by preparing five replicates at 10 and 100 mg/l of cyclohexylbenzene in water (Table 9).

Table 9. Correlation coefficients, limit of detection (LOD) and repeatability.

Compound	r ¹	LOD (µg/L)	RSD ² (%)
Cyclohexylbenzene	1.00	0.5	
10 mg/L			18
100 mg/L			17

For more detailed information on the analytical method and calculation of its performance consult Guillot et al., 2006.

References

Guillot, S., Kelly, M.T., Fenet, H., Larroque, M., 2006. Evaluation of solid phase microextraction as an alternative to the official method for the analysis of micro-pollutants in drinking water. *J Chromatogr A*. 1101: 46-52.

1.1.9. Di-2-ethylhexyl adipate (DEHA)

Principle of the test

The chemical di-2-ethylhexyl adipate (DEHA) is a plasticizer in widespread use. For example, it is frequently used in cling wraps, packaging and medical products. Furthermore, DEHA is one of the several high production volume chemicals that are under evaluation for potential environmental hazard and risk. The following protocol allows to analyse the concentration of DEHA (CAS number 103-23-1) in river water samples. The DEHA analytic quantification can be done using gas chromatography with a flame ionization detector (FID). Method validation is required for seawater samples.

Analytical procedure

1. Water samples should be collected in 10 L Pyrex® glass reagent bottles.¹
2. A sample of HPLC-grade water from an unopened bottle can be used as a control.

¹ To avoid potential contamination of samples with the plasticizer DEHA: 1) Before sampling, the bottles should be rinsed three times with lab grade acetone, distilled water, and HPLC-grade chloroform (total of nine washes); 2) Aluminum foil can be used as a barrier between plastic screw tops and the samples. Then, the bottles should be stored at 4 °C until extraction.

3. The pH of the samples should be reduced to 1.5 using 10 M of HCl and after that the samples should be extracted twice with 100 ml of HPLC-grade chloroform. The chloroform from the combined extracts should be evaporated and the residue should be re-dissolved in 1 ml of HPLC-grade chloroform.
 4. Samples should be then analysed using a Gas Chromatograph (GC) (e.g. a Varian CP-3800 GC) equipped with a flame ionization detector (FID).
 5. A CP-Sil 5 CB column (15 m x 0.53 mm) can be used.
 6. GC settings should be as follows: an injector temperature of 250 °C, an initial column temperature of 40 °C, a hold time of 2 min, a first temperature ramp rate of 10 °C per minute to 150 °C and a second temperature ramp rate of 20 °C per minute to a final temperature of 300 °C. The detector temperature should be 300 °C.
 7. Calibration curves should be obtained.
- Note: DEHA (99 %) should be used.
8. Identification of DEHA should be based on a comparison to the GC retention times.

Analytical performance

The 95 % confidence limit on the analysis of DEHA should be approximately 5 µg/l based on six samples.

References

- Barnabé, S., Beauchesne, I., Cooper, D.G., Nicell, J.A., 2008. Plasticizers and their degradation products in the process streams of a large urban physicochemical sewage treatment plant. *Water Res.* 42: 153-162.
- Horn, O., Nalli, S., Cooper, D., Nicell, J., 2004. Plasticizer metabolites in the environment. *Water Res.* 38: 3693–3698.
- Robillard, K.A., DuFresne, D.L., Gorsuch, J.W., Stubblefield, W.A., Staples, C.A., Parkerton, T.F., 2008. Aqueous Solubility and *Daphnia magna* Chronic Toxicity of Di(2-ethylhexyl) Adipate. *Bull Environ Contam Toxicol.* 80: 539-543.

1.1.10. 1-Dodecanol

Principle of the test

1-Dodecanol is a saturated 12-carbon fatty alcohol used in detergents, lubricating oils, pharmaceuticals, etc. The following protocol is suitable to determine the concentration of 1-dodecanol (CAS number 112-53-8, UN number 3077) in environmental river water samples. To this end, water samples (low volume) should be subjected to solid phase extraction (SPE) combined with dispersive liquid-liquid microextraction (DLLME) in order to preconcentrate this compound. Then, the extracted samples should be subjected to a fast derivatisation procedure performed at room temperature and assessed with the use of high performance liquid chromatography (HPLC)-tandem mass spectrometry (MS). Method validation is required for seawater samples.

Analytical procedure

1. Water samples should be collected to clean glass bottles pre-rinsed with the sample.
2. After collection, samples must be subjected to solid-phase extraction (SPE).
 - 2.1 1-dodecanol should be isolated and enriched using SPE.
 - 2.2 Polystyrene-divinylbenzene cartridge - Strata SDB-L (100 mg, 1 ml, 100 μm , 260 \AA) should be conditioned with 8 ml of methanol and 8 ml of water.
 - 2.3 Before the cartridge dried, a 30 ml of water sample should be applied at a speed of ca. 2 ml min^{-1} .
 - 2.4 The cartridge should be dried with vacuum.
 - 2.5 After drying is complete, the analyte should be eluted with 2 ml of ethanol.
3. A dispersive liquid-liquid microextraction procedure should be done.
 - 3.1 A extracting solvent (80 μl of trichloroethylene) should be added to the dispersing solvent (the ethanolic eluate from SPE) placed in a 15 ml glass test tube with a conical bottom.
 - 3.2 Then, pure water² (6 ml) must be added quickly.

Note: In this stage, the extraction solvent disperses into the water as very fine droplets and a cloudy solution is formed in the test tube.
 - 3.3 After that, the mixture should be centrifuged for 10 min at 4500 rpm.
 - 3.4 The dispersed fine particles of extraction phase must sediment in the bottom of the test tube.
 - 3.5 The sedimented phase (70 μl) should be withdrawn with a 100 μl micro-syringe.
 - 3.6 The extract should be evaporated with a gentle nitrogen purge at room temperature and derivatised.
4. The evaporated extract should be derivatised with 28 μl of a mixture consisting of 1 μl of 1-naphthoyl chloride (NC) and 1 μl of pyridine³ in 1 ml of acetonitrile (use smaller vials!).
 - 4.1 The reaction should be carried out for 20 min at room temperature.
 - 4.2 The mixture should be manually shaken every 5 min.
 - 4.3 Then, 2 μl of methanol should be added to the sample in order to quench the reaction; the sample should be left for 10 min.
5. HPLC analysis with mass spectrometric detection should be the next step. Hence, the sample should be injected into HPLC column for analysis.
 - 5.1 A chromatographic system (e.g. chromatographic system UltiMate 3000 RSLC from Dionex) should be used.
 - 5.2 Inject 5 μl of the sample into a column (e.g. Hypersil GOLD column) (100 mm x 2.1 mm I.D.; 1.9 μm) with a 2.1 mm I.D. filter cartridge (0.2 μm).
 - 5.3 The mobile phase shall consist of 5 x 10⁻³ mol l^{-1} of ammonium formate in water (A) and acetonitrile (B) at a flow rate of 0.3 ml min^{-1} at 35 $^{\circ}\text{C}$.

² Water can be prepared by reverse osmosis (e.g. in a Demiwa system from Watek) followed by double distillation from a quartz apparatus.

³ Pyridine is used as a derivatisation catalyser.

- 5.4 Gradient elution should be performed by linearly increasing the percentage of organic modifier from 80 to 90 % in 10 min and then it should be maintained at 90 % for 5 min.
- 5.5 A pre-run time of 4 min should be done before the next injection.
- 5.6 The chromatographic system should be connected to the mass spectrometer (e.g. API 4000 QTRAP triple quadrupole mass spectrometer).
- 5.7 The LC column effluent should be directed to the electrospray ionisation source (Turbo Ion Spray).
- 5.8 The Turbo Ion Spray source should operate in positive ion mode.
- 5.9 The dwell time for each mass transition detected in the MS/MS multiple reaction monitoring (MRM) mode should be set to 100 ms.
- 5.10 1-dodecanol should be detected using the following settings for the ion source and mass spectrometer:
- Curtain gas - 20 psi;
 - Nebuliser gas - 40 psi;
 - Auxiliary gas - 50 psi;
 - Temperature - 400 °C;
 - Collision gas medium and;
 - Ion spray voltage - 5500 V.
- 5.11 The $[M+H]^+$ adducts of 1-dodecanol should be used as precursor ions.

Analytical performance

The limit of detection (LOD) of the method (SPE-DLLME sample preparation and LC-MS/MS analysis) should be 1.2 ng L⁻¹.

Precision (RSD) of the method for four water samples should be 1.7 %.

More information on the 1-dodecanol analytical quantification:

- Method reported above: consult Zgoła-Grzésekowiak and Grzésekowiak (2012).
- **Other method:** Use LC-MS/MS (with electrospray ionization) after 1-dodecanol derivatization (with phenyl isocyanate (PIC)) in combination with liquid-liquid extraction (using ethyl acetate) (LOD = 0.005 µg l⁻¹) - consult Zembrzuska J. (2017).

References

Zembrzuska, J., 2017. Determination of Dodecanol and Short-Chained Ethoxylated Dodecanols by LC–MS/MS (with Electrospray Ionization) After Their Derivatization (with Phenyl Isocyanate). *J Surfactants Deterg.* 20: 1421-1432.

Zgoła-Grzésekowiak, A., Grzésekowiak, T., 2012. Solid-phase extraction combined with dispersive liquid–liquid microextraction, fast derivatisation and high performance liquid chromatography-tandem mass spectrometry analysis for trace determination of short-chained dodecyl alcohol ethoxylates and dodecyl alcohol in environmental water samples. *J Chromatogr A.* 1251: 40-47.

1.1.11. 4-Nonylphenol

Principle of the test

Nonylphenols (NPs) are by far the most important alkylphenols (APs), constituted by an alkyl chain with 9 C located at either *ortho*- (2-NP), *meta*- (3-NP), or mainly *para*- (4-NP) position on the phenolic ring. These compounds are the degradation products of nonylphenol ethoxylates (NPEs), one of the most common non-ionic surfactants utilised in cleaning products and detergents. Furthermore, NPs are used as pesticides, as a monomer in phenol/formaldehyde resins and mainly as plasticizers, etc.

4-NP, an endocrine disrupting chemical (EDC), has been included in different EU and international regulations to preserve the environment and protect human health because of its persistence in the environment, its moderate bioaccumulation in organisms and its toxicity. Therefore, the EU has included 4-NP on the list of priority hazardous substances for surface water and its chronic and acute environmental quality standards are 0.3 and 2 $\mu\text{g L}^{-1}$, respectively (Directive 2013/39/EU). In USA, EPA recommends that 4-NP concentration should not exceed 1.7 $\mu\text{g L}^{-1}$ in saltwater.

The simple, fast, sensitive and environmental friendly analytical method described below is suitable to determine the concentration of 4-nonylphenol (CAS number 104-40-5, UN number 3145) in seawater samples. To this end, a small volume of seawater sample (30 ml) should be subjected to dispersive liquid-liquid microextraction (DLLME) using only 100 μl of 1-octanol in order to preconcentrate the compound. This procedure should be combined with liquid chromatography–electrospray ionization tandem mass spectrometry in negative mode (LC–ESI-MS/MS).

Analytical procedure

1. Seawater samples should be collected in amber glass containers and stored at 4 °C before the analysis. Note: Samples should be assessed within five days of their sampling due to the low stability of APs.
2. Then, aliquots of 30 ml of seawater samples should be extracted by dispersive liquid–liquid microextraction (DLLME) as follows:
 - 2.1 100 μl of 1-octanol (grade HPLC 99 %) should be added as extractant solvent.
 - 2.2 The mixture should be vigorously shaken using an agitation plate (e.g. agitation plate Vibrax-VXR by IKA) for 5 min at 1200 rpm.
 - 2.3 Separation of two phases occurs upon centrifugation (using Eppendorf) at 3500 rpm for 3 min.
 - 2.4 The fine droplets of 1-octanol should be collected and then the volume should be adjusted to 1 ml with methanol due to the immiscibility of the 1-octanol with the LC mobile phase.
 - 2.5 The extract should pass through a 0.2 μm syringe filter of PTFE before LC injection in order to remove any solid particles that might interfere in the analysis and damage the equipment.
3. After that, samples should be analysed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

- 3.1 LC analyses should be performed using a HPLC system (e.g. an Agilent HP-1200 Series LC system) equipped with an autosampler (volume injected should be 25 μ l), a binary solvent pump and a thermostated column oven.
 - 3.2 The chromatographic separation should be carried out with a column (e.g. Hypersil Gold C18) (150 mm \times 2.1 mm), 3 μ m, using as mobile phase A (water) and B (methanol) with 0.05 % of ammonia (30 %) as modifier.
 - 3.3. A 14 min gradient should be performed as follows: 20 % B (1 min) to 100 % B in 4 min; this percentage should be maintained during 7 min and returned to initial conditions in 2 min.
 - 3.4 The system should be re-equilibrated for 7 min between runs.
 - 3.5 The flow rate should be 0.25 ml min⁻¹ and the oven temperature should be set at 40 °C.
 - 3.6 The LC system must be coupled to a mass spectrometer with a triple quadrupole detector and an APCI (atmospheric pressure chemical ionization) / ESI (electrospray ionization) source (e.g. API 3200). All compounds should be detected with ESI interface operating in negative mode.
 - 3.7 For quantification, multiple-reaction monitoring (MRM) should be chosen as an acquisition mode because it allows high selectivity and sensitivity.
 - 3.8 Four identification points (one precursor ion [M-H]⁻ and two products ions) should be required for an appropriate confirmation of 4-nonylphenol (for LC-MS/MS analysis).
 - 3.9 The relation between the transitions (MRM ratio) should also be used as a criterion for the identification in samples analysis.
4. Procedural blanks should be frequently checked (analysed in triplicate) and kept under control. If the blank analysis reveals the presence of 4-nonylphenol, such concentration should be subtracted from the samples in order to avoid overestimations in the results.

Notes: Do not use detergents and plastic material in order to reduce blank problems! Carefully wash all the glassware with acetone, Milli-Q water and methanol before use!

Analytical performance

Repeatability (n=7), intermediate precision (n=10) and recoveries (n=7) should be < 10 %, \leq 7 %, and around 91–103 %, respectively.

Quantitation limit of the method (MQL) should be 0.005 μ g L⁻¹.

More information (e.g. preparation of standard solutions using 4-n-nonylphenol 99.9 % purity (\pm 0.5 % tolerance), LC-MS/MS variables, etc.): consult Salgueiro-González et al. (2012).

References

Salgueiro-González, N., Concha-Graña, E., Turnes-Carou, I., Muniategui-Lorenzo S., López-Mahía, P., Prada-Rodríguez, D., 2012. Determination of alkylphenols and

bisphenol A in seawater samples by dispersive liquid–liquid microextraction and liquid chromatography tandem mass spectrometry for compliance with environmental quality standards (Directive 2008/105/EC). *J Chromatogr A*. 1223: 1-8.

Tato, T., Salgueiro-González, N., León, V.M., González, S., Beiras, R., 2018. Ecotoxicological evaluation of the risk posed by bisphenol A, triclosan, and 4-nonylphenol in coastal waters using early life stages of marine organisms (*Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Acartia clausi*). *Environ Pollut*. 232: 173-182.

1.1.12. Analysis of tetrachloroethylene and trichloroethylene

Principle of the test

Tetrachloroethylene (C₂Cl₄) and trichloroethylene (C₂HCl₃) are halogen-containing volatile organic compounds (i.e. halocarbons). They are anthropogenically produced halocarbons widely used as refrigerants, solvents, dry cleaning fluids, metal surface degreasing agents, etc. In addition, these chlorinated compounds are environmentally significant because of their toxicity, thus posing hazards to marine ecosystems and human health.

The following protocol allows to determine the concentration of two HNS considered priority - tetrachloroethylene (CAS number 127-18-4, UN number 1897) and trichloroethylene (CAS number 79-01-6, UN number 1710) - in seawater. Such concentrations should be measured using a purge and trap system coupled to a gas chromatograph (GC) with an electron capture detector (ECD).

Analytical procedure

1. Seawater samples can be collected⁴ (e.g. using Niskin bottles (8 L) mounted to a Seabird CTD Rosette) and immediately assessed.
2. The concentrations of tetrachloroethylene and trichloroethylene in seawater should be measured using a purge and trap system coupled to a gas chromatograph (GC) with an electron capture detector (ECD) as follows:
 - 2.1 100 ml of seawater samples should be drawn into a glass syringe from the Niskin bottle using a silicone tube without contact with ambient air.
 - 2.2 Then, the sample should be injected into a purge chamber and purged with ultrapure nitrogen for 12 min at a flow of 100 ml min⁻¹.
 - 2.3 The gas flow should be dried through a glass tube containing magnesium perchlorate (MgClO₄).
 - 2.4 Tetrachloroethylene and trichloroethylene should be concentrated in an empty stainless steel tube (length: 30 cm; i.d.: 0.8 mm) immersed in liquid nitrogen (-196 °C).

⁴ For information on seawater sampling - consult He et al. (2013a,b).

- 2.5 After thermodesorption in boiling water (100 °C), halocarbon compounds should be introduced into the GC column (e.g. DB-624, length: 60 m; i.d.: 0.53 mm; film thickness: 3 mm) by the carrier gas (N₂) at the flow rate of 5.6 ml min⁻¹.
- 2.6 The GC temperature program should be initially maintained at 45 °C for 7 min, then raised to 90 °C at the rate of 5 °C min⁻¹ and finally to 200 °C at the rate of 10 °C min⁻¹. At the end, keep the temperature at 200 °C for 5 min. Note: The total run time should be 32 min.
- 2.7 The trace gases must be identified and quantified according to the retention times and peak areas of the compounds in calibration standards.
- 2.8 To calibrate the concentrations of the trace gases, the commercially available calibration standards should be diluted twice with methanol to make working standards.
- 2.9 After that, the working standards should be subjected to the same procedure as the real seawater samples.
Note: Calibrations for seawater measurements should be done each day.
- 2.10. Blanks should be established each day by running the system without a water sample or by repeatedly purging a water sample so that it is free of the dissolved target compounds.

Analytical performance

The method detection limit (MDL) of the compounds (calculated from the data of seven replicate measurements of low concentration standards) should be between 0.1 and 0.5 pmol L⁻¹.

The precision (defined as relative standard deviations of quadruplicate analyses of the seawater samples) should be in the range of 7-15 % in the sample analysis.

The overall accuracy of the method (determined by repeated analyses of low concentration standards (n = 7)) should be between 5 % and 18 % for the target compounds.

More information on the analytical quantification of tetrachloroethylene and trichloroethylene:

- Method reported above: consult He et al. (2013a).
- **Other method:** Use static headspace sampling and gas chromatography with electron capture detection (HS-GC-ECD) (LODs = 0.002 and 0.005 µg l⁻¹, respectively) - consult Li et al. (2016).

References

He, Z., Yang, G.-P., Lu, X.-L., Zhang, H.-H., 2013a. Distributions and sea-to-air fluxes of chloroform, trichloroethylene, tetrachloroethylene, chlorodibromomethane and bromoform in the Yellow Sea and the East China Sea during spring. *Environ Pollut.* 177: 28-37.

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Li, T., Guo, Y., Hu, H., Zhang, X., Jin, Y., Zhang, X., Zhang, Y., 2016. Determination of volatile chlorinated hydrocarbons in water samples by static headspace gas chromatography with electron capture detection. *J Sep Sci.* 39: 358-366.

Yang, G.-P., Lu, X.-L., Song, G.-S., Wang, X.-M., 2010. Purge-and-trap gas chromatography method for analysis of methyl chloride and methyl bromide in seawater. *Chinese J Anal Chem* 38: 719-722.

1.1.13. m-Cresol

Principle of the test

m-cresol is a naturally occurring phenolic compound and is used for the synthesis of pesticides, plastics, pharmaceuticals and dyes. This chemical is used as a solvent, reagent in coal tar refining, disinfectant, chemical intermediate in the production of resins and polymeric materials and also in endodontic treatments in dentistry. However, m-cresol can pose a potential threat to aquatic and human life when introduced into the environment. For example, excessive amounts of m-cresol can cause severe damage to heart, liver, lungs and kidneys. It can also lead to coma paralysis, and even death to the human beings. Furthermore, it is proved as carcinogenic.

The following protocol allows to determine the concentration of m-cresol (CAS number 108-39-4, UN number 2076) in seawater. To this end, seawater samples should be subjected to solid phase extraction (SPE) combined with the use of high performance liquid chromatography (HPLC).

Analytical procedure

1. After collection, seawater samples (100 ml) should be adjusted to pH = 3.0 with phosphoric acid (H_3PO_4) and filtered through a 0.45- μm nylon membrane.
2. Then, the samples should be extracted through a solid-phase extraction (SPE) using an appropriate extraction column (e.g. Bond Elut C_{18} (500 mg)) as follows:
 - 2.1 Before sampling, each column should be conditioned with 5 ml of methanol and then with 15 ml of water, pH = 3.0 (acidified with H_3PO_4).
 - 2.2 Different volume samples should be filtered through the sorbent bed under reduced pressure by using the Vat Master-10 sample processing station (International Sorbent Technology) at a flow-rate of approximately 5 ml/min, and dried with air under the same conditions for 5 min.
 - 2.3 m-cresol should be eluted from the column with 1.5 ml of a mixture water-acetonitrile (1: 1, v/v).

Note: Methanol must not be used since it gives broad peaks in the chromatogram.

3. HPLC analysis should be the next step.

3.1 A liquid chromatograph (e.g. Hewlett-Packard Model 1040A liquid chromatograph) equipped with a diode array detector (DAD) (e.g. Hewlett-Packard 1040M Series II diode array detector) linked to a data system (e.g. Hewlett-Packard Chem-Station) should be used for data acquisition and storage. The system should be coupled to a quaternary pump and an automatic injector (e.g. Hewlett-Packard, 1050 Series).

3.2 The column can be a HP-Lichrospher 100 RP-18 (5 μm) (250 x 4 mm I.D.).

3.3 The detector should be set to record the chromatogram at six different analytical signals.

3.4 The assays should be performed at room temperature.

3.5 A water-acetonitrile mixture (64:36, v/v) should be used as mobile phase.

3.5.1 All the solutions should be prepared daily, filtered through a 0.45- μm nylon membrane and degassed with helium before use.

3.5.2 The flow-rate should be set at 1 ml/min.

3.6 Inject 50 μL of each sample.

4. If necessary, the H-point standard additions method (HPSAM) can be used to resolve highly overlapped peaks in liquid chromatography.

Note: The method uses the areas obtained at two previously selected wavelengths as analytical signals.

Notes (for preparation of reagents):

- 1) All the reagents should be of analytical-reagent grade, and acetonitrile should be LC grade.
- 2) Water should be distilled, deionized and filtered through 0.45- μm nylon membranes.
- 3) Standard solutions (1000 ppm) should be prepared by dissolving *m*-cresol in water and stored in borosilicate-glass vessels in the refrigerator.

Analytical performance

The detection limit of the method should be < 1 ppb.

20 ppb of *m*-cresol can be quantified without error.

References

Ghai, M., Narula, P., Kaur, V., Singh, R., 2015. Imprinted silica nanoparticles coated with *N*-propylsilylmorpholine-4-carboxamide for the determination of *m*-cresol in synthetic and real samples. *J Sep Sci.* 38: 3442-3449.

Reig, F.B., Falcó, P.C., Andrés, J.V., 1996. H-Point standard additions method for resolution of overlapping chromatographic peaks with diode array detection by using area measurements. Determination of phenol and cresols in waters. *J Chromatogr A.* 726: 57-66.

1.1.14. Nitrobenzene

Principle of the test

Nitrobenzene (NB) is one of the main carcinogenic nitroaromatic compounds and has been widely used for the preparation of dyes, explosives pharmaceuticals and pesticides. In recent years, considerable attention has been given to the release of nitrobenzene into the aquatic environment due to their toxicity, persistence, and accumulation in food chains. Thus, this priority hazardous compound can represent a threat to aquatic environment and human health. The following protocol allows to determine the concentration of nitrobenzene (CAS number 98-95-3, UN number 1662) in seawater. To this end, seawater samples should be subjected to solid phase extraction (SPE) combined with the use of gas chromatography (GC) - mass spectrometry (MS).

Analytical procedure

1. After collection⁵, seawater samples (20 L) should be filtrated and extracted (e.g. using a filtration/extraction unit).
 - 1.1 High capacity filtration should be achieved by the use of glass fibre filter candles (exclusion size: 0.5 µm) coupled online to the solid-phase extraction (SPE) unit.

Notes:

 - a) Prior to application, glass fibre filter candles should be cleaned by heating them for 72h at 693K (420 °C) followed by duplicate Soxhlet extraction (200 ml of *n*-hexane/ethyl acetate, 6h);
 - b) Glass fibre filter disks GF/C must have 47 mm of diameter and 1.2 µm of exclusion size.
 - 1.2 The extraction should be done through a polymeric sorbent (Bakerbond SDB-1 (styrene-divinylbenzene co-polymer, particle size: 40-120 µm, pore size: 27 nm, surface area: 1060 m²/g); 2g) that should be packed in a glass cartridge (inner diameter 45mm).
 - 1.3 The flow rate applied should be 500 ml/min.
 - 1.4 Elution should be performed with 90 ml of ethyl acetate followed by 50 ml of a mixture of *n*-hexane/ethyl acetate (4:1 v/v).
 - 1.5 Eluates should be condensed by rotary evaporation to a final volume of approximately 100 µl after addition of *iso*-octane as a keeper.
 - 1.6 These extracts should be measured without further clean-up.
2. Then, samples should be assessed with the use of gas chromatography (GC) - mass spectrometry (MS).
 - 2.1 A GC (e.g. Varian 3400 GC) equipped with a split/splitless injector (e.g. injector 1075) (60 s splitless, 523K (250 °C)) and an appropriate column (e.g. DB5-MS column) (length 30 m, I.D. 0.25 mm, film thickness 0.25 µm) must be used.

⁵ For information on seawater sampling - consult Weigel et al. (2005).

- 2.2 The carrier gas should be helium 5.0 (75 kPa) and the transfer-line should be held at 523K (250 °C).
- 2.3 The GC should run with an autosampler (e.g. A 200 SE autosampler) (inject 2 µl of volume).
- 2.4 Standard temperature programmes should be 333 K (60 °C) [2 min] → (7 K/min) → 533 K (260 °C) [20 min] and 333 K (60 °C) [2 min] → (10 K/min) → 533K (260 °C) [20 min] for recovery study.
- 2.5 The gas chromatograph must be coupled to a Magnum ITD ion trap mass spectrometer which should operate under the following conditions:
- EI-ionisation at 70 eV;
 - Manifold temperature 473 K (200 °C);
 - Emission current: 10 µA;
 - Scan range: 100-420 amu.

Analytical performance

The recovery rate ($n = 4$, spiking level = 5 ng/L) \pm standard deviation (SD) should be $44 \pm 13 \%$ (for the extraction of simulated seawater (20 L)*).

The limit of quantification (LOQ, $s/n = 9$) should be 0.18 ng/L (for the extraction of simulated seawater (20 L).*

* Simulated seawater should be prepared by dissolution of 280 g of NaCl and 77 g of MgSO₄ in 10 L of deionised water which corresponds to a salinity of 35‰ when Ca-ions are replaced by Mg. The pH should be adjusted to 8.3 by the addition of NaHCO₃. The obtained artificial seawater should be spiked with 1 ml/10 L of the standard solution in acetone.

(Note: Inorganic salts for the preparation of simulated water should be cleaned and dried by heating them overnight at 873 K (600 °C), except for NaHCO₃: 523 K (250 °C).)

References

- Weigel, S., Bester, K., Hühnerfuss, H., 2001. New method for rapid solid-phase extraction of large-volume water samples and its application to non-target screening of North Sea water for organic contaminants by gas chromatography–mass spectrometry. *J Chromatogr A*. 912: 151-161.
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- Zhang, G.-J., Zhou, X., Zang, X.-H., Li, Z., Wang, C., Wang, Z., 2014. Analysis of nitrobenzene compounds in water and soil samples by graphene composite-based solid-phase microextraction coupled with gas chromatography-mass spectrometry. *Chin Chem Lett*. 25: 1449-1454.

1.1.15. Acrylonitrile

Principle of the test

Acrylonitrile is considered a priority HNS highly transported in Atlantic European waters, being used extensively in the production of plastics, resins, synthetic fibres and rubbers. Several acrylonitrile spills have been reported worldwide (e.g. Alessandro Primo which carried 550 tons of acrylonitrile and sank in the Adriatic Sea near the coast of Italy). This substance has obtained worldwide attention in recent years based essentially on its moderate toxicity to aquatic organisms and the fact that it is a potential human carcinogen.

The following protocol allows to determine the concentration of acrylonitrile (CAS number 107-13-1) in seawater. The acrylonitrile analytic quantification should be done by high performance liquid chromatography (HPLC).

Analytical procedure

1. Seawater samples should be collected in duplicate and then directly assessed by high performance liquid chromatography (HPLC) with a photodiode array detector (DAD). Therefore, a liquid chromatographic system must be used (e.g. a Hitachi LaChrom Elite HPLC, constituted with a L-2130 quaternary pump, an in line degasser, a L-2455 photodiode detector, and a L-2200 autosampler).
2. Separations can be performed with a 250 x 4.6 mm (length x i.d.), 5 µm particle, Purospher® STAR RP-18e analytical column (Merck) and a 4 x 4 Chmm i.d., 5 µm particle, guard column with the same packing material.
3. The mobile phases (acetonitrile (A) and water (B)) should have a flow rate of 1 ml min⁻¹ and the injection volume can be set to 50 µl.
4. The acrylonitrile elution can be done in an isocratic mode (50 % A and 50 % B) maintaining the temperature of the column at 30 °C.
5. Acrylonitrile should be monitored at a wavelength of 195 nm.

Analytical performance

The detection and quantification limits (LOD and LOQ) should be 25 and 75 µg L⁻¹, respectively.

More information on the acrylonitrile analytical quantification can be found in U.S. EPA Method 8316, 1994.

References

U.S. EPA Method 8316, "Acrylamide, Acrylonitrile and Acrolein by High Performance Liquid Chromatography (HPLC), Revision 0." U.S Environmental Protection Agency, 1994.

Neuparth, T., Capela, R., Rey-Salgueiro, L., Moreira, S.M., Santos, M.M., Reis-Henriques, M.A., 2013. Simulation of a Hazardous and Noxious Substances (HNS) spill in the marine environment: Lethal and sublethal effects of acrylonitrile to the European seabass. *Chemosphere* 93: 978–985.

Chapter 2. Biological assessment

2.1. Bioassays

Soares, J; Oliveira, H.; Santos, M.M.

This book includes bioassays for both water and whole-sediment matrices. Tier 1 screening of water systems should consist of a battery of a minimum of three tests from three trophic levels (fish, crustaceans, algae) in order to be representative of the entire ecosystem under investigation. The test battery should consist of a combination of short term acute and prolonged (sub) lethal tests (e.g. development analysis) in order to cover the most sensitive endpoints/species. Suggestions for test protocols are made in accordance with recommendations from GESAMP and the OSPAR Commission. The majority of the guidelines for the water matrix, describe procedures for water samples that are chemically contaminated at the laboratory to evaluate the substance toxicity. Nevertheless, these can also be applied to environmental samples, used either as received or using a range of concentrations (e.g. see the oyster embryo bioassay). Additionally, the guidelines described for the water matrix are also relevant, and can be applied, for sediment pore-water and sediment seawater elutriates. For information on how to prepare sediment elutriates and pore water extracts consult Davoren et al. 2005. As an example of a test battery that could be employed the OSPAR Commission (2007) recommends the following species to be included for marine water samples: 1) Copepods (such as *Tisbe battagliai*, *Acartia* sp.): 48 h exposure, endpoint mortality, (2) Bivalves (*Crassostrea gigas*, *Mytilus* spp): 24 h embryo exposure, endpoint percent net response, (3) Sea urchin (*Paracentrotus lividus*): 24 h embryo exposure, endpoint percent normal development and larval length. For whole-sediment tests the amphipod crustaceans such as *Corophium* (10-d LC50) and polychaete worms such as *Arenicola* (10-d LC50) are the species of choice.

When choosing the adequate test for the chemical to be analyse it must be taken in consideration its physico-chemical proprieties. The octanol-water partition coefficient (K_{ow}) of a substance is a measure of its hydrophobicity and a good indicator of its partitioning potential in the organic fraction of the sediment (K_{oc}) in aquatic environments. According to the EC guidance document, a rule of thumb is that compounds with a $\log K_{ow} > 5$ should primarily be found in sediments, while compounds with a $\log K_{ow} < 3$ should primarily be found in the water matrix. For substances with a $\log K_{ow}$ between 3 and 5, the sediment matrix is an optional matrix to analyse depending on the degree of contamination.

References

EC Guidance document no. 25, 2010. On chemical monitoring of sediment and biota under the Water Framework Directive. Common implementation strategy for the Water Framework Directive (2000/60/EC). Technical Report 2010.2991. European Commission.

Davoren, M., Shúilleabháin, S.N., O'Halloran, J., Hartl, M.G., Sheehan, D., O'Brien, N.M., van Pelt, F.N., Mothersill, C., 2005. A test battery approach for the ecotoxicological evaluation of estuarine sediments. *Ecotoxicol.* 14(7): 741-755.

OSPAR Commission 2007. Background document on biological effects monitoring techniques. Assessment and Monitoring Series. Available at: www.ospar.org.

2.1.1. Test matrix: *Water (also relevant for sediment pore-water and sediment seawater elutriates)*

2.1.1.1. Fish Acute Toxicity Test

Based on OECD Test Guideline Series No. 203.

Principle of the test

This guideline prescribes a test using fish as test organisms to develop data on the acute toxicity of chemicals. The fish are exposed to the test substance, preferably for a period of 96 hours (h). Mortalities are recorded at 24, 48, 72 and 96 h and the concentrations which results in 50 % of fish mortality (LC50) are determined where possible.

Test conditions

Equipment

- Oxygen meter;
- Equipment for determination of water hardness;
- Apparatus for water temperature control;
- Tanks of chemically inert material.

Species

1. Species selection is at the discretion of the testing laboratory. Several important criteria should be taken into consideration: availability throughout the year, ease of maintenance, convenience for testing and any relevant economic, biological or ecological factors. This is a flexible guideline allowing the use of many freshwater and marine species. For chemicals test in salt water a small estuarine fish, the sheepshead minnow (*Cyprinodon variegatus*) has been found suitable. Furthermore, the three-spined stickleback (*Gasterosteus aculeatus*), that live in both marine and fresh waters, is increasingly used as a test organism and it is ecologically relevant for several European countries.

Acclimatisation

2. All fish must be held in the laboratory at least 12 days before they are used for testing. At least 7 days before testing they should be held in the water of the quality to be used under the following conditions:

- a. Light: 12-16 h of photoperiod daily;
- b. Oxygen concentration: at least 80 % of saturation value;

- c. Feeding: 3 times per week or daily until 24 h of the test is started;
- d. Temperature: according to the species.

Following a 48 h settling-in period, mortalities are recorded and the following criteria applied:

- a) If mortalities exceed 10 % in 7 days, the entire batch is rejected;
- b) Mortalities between 5-10 % of population, acclimatisation continued for 7 additional days;
- c) Mortalities of less than 5 % of population, acceptance of batch.

Water

3. Good quality natural water or reconstituted water is preferred, although drinking water (dechlorinated, if necessary) may also be used. For salt water testing, natural or synthetic salt water may be used as the test medium. If natural seawater is used, it should be collected from an uncontaminated area. If synthetic salt water is used, it should be prepared by dissolving reagents of known analytical grade, or a commercially available formulation, in distilled or deionised water.

Natural or synthetic salt water used for solutions dilution and in the toxicity test should be passed through a filter effective to $\leq 0.2 \mu\text{m}$ to remove suspended particles, organisms and facultative pathogens from the water. Also, the water should be intensively aerated before use (≥ 90 % oxygen saturation), and aging for one to two weeks might be desirable.

Test solutions

4. Test solutions of the chosen concentrations are prepared by dilution of a stock solution in either with Milli-Q water (freshwater testing) or saltwater (saltwater testing). In the case of substance with low water solubility, stock solutions may be prepared by ultrasonic dispersion or other suitable physical means. If necessary, vehicles (e.g. organic solvents) of low toxicity to fish may be used, in concentrations that should not exceed 100 mg/L. When vehicles are used, an additional control should be exposed to the same concentration of the vehicle as that used in the most concentrated solution of the test substance.

5. The test should be carried without adjustment of pH, otherwise it is advisable to repeat the test, adjusting the pH of the stock solution to that of tank water before addition of the test substance. Adjustment should be made without significant alterations of the stock solution concentration or chemical precipitation of the test substance. HCl and NaOH are preferred.

Test procedure

Conditions of exposure

- 1. Duration: 96 h;
- 2. Loading: maximum loading of 1.0 g of fish/L for static or semi-static tests; for flow-through systems a higher loading can be accepted;
- 3. Light: 12-16 h photoperiod daily;
- 4. Temperature: appropriate to the test species and constant within a range of 2 °C;
- 5. Oxygen concentration: ≥ 60 % of the air saturation value. Aeration can be used provided that it does not lead to a significant loss of the test substance;

6. Feeding: none;
7. Disturbance: disturbances that may change the behaviour of the fish should be avoided;
8. Water parameters: freshwater testing - 10-250 mg CaCO₃/L, pH 6-8.5, conductivity ≤ 10 μScm⁻¹; salt water testing - pH 8.0, salinity 20-30‰;
9. Measurement of pH, dissolved oxygen and temperature should be carried out at least daily.

Exposure

10. A minimum of 7 fish should be exposed to at least 5 concentrations in a geometric series with a factor preferably not exceeding 2.2. One blank and, if relevant, one control containing the solubilizing agent are run in addition to the test series, with an equal number of fish.

Note: A range-finding test properly conducted before the definitive test enables the choice of the appropriate concentration range.

Observations

11. The fish are inspected at least at 24, 48, 72 and 96 h and mortality is recorded. Fish are considered dead if there are no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Dead fish are removed. Records of visible abnormalities are recorded (e.g. loss of equilibrium, swimming behaviour, respiratory function, pigmentation, etc.). Observations at 3 and 6 h after the test start are desirable.

Results

1. The cumulative percentage mortality for each exposure period is plotted against concentration on a logarithmic scale;
2. The LC₅₀ is calculated by normal statistical procedures for the appropriate exposure period, if possible (confidence limits (p=0.95));
3. A maximum concentration causing no mortality within the period of test and a minimum concentration causing 100 % mortality within the period of the test should be determined;
4. Where the data are inadequate for the use of standard methods of calculating the LC₅₀, the maximum concentration causing no mortality within the period of test and the minimum concentration causing 100 % mortality within the period of the test should be used as an approximation of the LC₅₀ (this being considered the geometric mean of these two concentrations).

Validity of the test

1. The mortality in the controls should not exceed 10 % (or one fish if less than 10 are used) at the end of the test;
2. Constant conditions should be maintained as far as possible throughout the test;
3. The dissolved oxygen concentration should be at least 60 % of the air saturation value throughout the test;

4. Evidence that the concentration of the substance being tested has been satisfactorily maintained and preferably, it should at least 80 % of the nominal concentration. Otherwise, results should be based on the measured concentration.

References

GESAMP (IMO/FAO/UNESCO-IOC/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection), 2014. Revised GESAMP Hazard Evaluation Procedure for Chemical Substances Carried by Ships, 2nd edition. Rep. Stud. GESAMP No. 64, 126 pp.

McGowan, T., Sheahan, D., Cunha, I., Oliveira, H., Santos, M.M, 2013. ARCOPOLplus activity 2, task 2.2.1 Determination of acute and chronic toxicity of priority HNS upon representatives of different marine plant and animal taxa.

OECD, 1992. Test Guideline Series No. 203: Fish, acute toxicity test. Organisation for Economic Co-operation and Development, Paris.

2.1.1.2. Determination of acute lethal toxicity to marine copepods

Principle of the test

This guideline prescribes a test using a harpacticoid copepod (*Tisbe battagliai*) as test organism to develop data on the acute toxicity of chemicals. Newly hatched larvae (termed nauplii), aged less than 24 hours at the start of the test, are exposed in microwell test chambers to seawater (*i.e.*, control) and to the test chemical added to seawater at a range of concentrations. The test duration is of 48 hours (h). Mortalities are recorded at 24 and 48 h and the concentrations which results in 50 % of copepods mortality (LC50) are determined at 48 h where possible.

Static and renewal microplate tests might not be applicable to materials that have a high oxygen demand, are highly volatile, are rapidly transformed (biologically or chemically) in aqueous solutions, or are removed from test solutions in substantial quantities by the test chambers or organisms during the test.

Test conditions

Equipment

- Oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volume (*e.g.*, ≤ 10 -mL) samples);
- pH meter;
- Apparatus for water temperature control;
- Apparatus for water salinity control;
- 125 μm mesh sieve;

- 62 µm nylon mesh;
- Polystyrene 24-well microplates;
- Crystallising dish;
- Pasteur pipettes;
- 500-µL glass syringe;
- Graduated glass capillary pipette;
- Test vessels;
- Dissecting stereomicroscope.

Note: Polystyrene is not well suited to testing with hydrophobic organic chemicals because of non-specific plastic:organic binding, which can reduce chemical bioavailability to copepods. Therefore, microplates with microwells pre-coated with glass or polyacrylamide are better suited for copepod testing of hydrophobic chemicals than microplates with naked polystyrene wells.

Species

1. Copepods belonging to the genus *Tisbe* are particularly useful for risk assessment due to their small size, relatively short life cycle and the ease of continuous culture. *Amphiascus tenuiremis*, *Nitocra spinipes* and *Acartia tonsa* have also been proven to be valuable test organisms for the assessment of marine contaminants in temperate ecosystems. It is recommended that test animals be obtained from already established laboratory cultures, derived from a healthy stock.

Acclimatisation

2. All copepods in a test should be from the same brood stock. The two previous generations should have been raised from birth using the same seawater, photoperiod and temperature as used in the toxicity test. This will not only acclimate the copepods, but will also demonstrate the acceptability of the seawater and handling procedures before the test is begun. Additionally, copepod stock cultures should be fed a 1:1:1 mixed algal cell suspension, in excess, of a chlorophyte (for example, *Dunaliella tertiolecta*), a chrysophyte (for example, *Isochrysis galbana*), and a diatom (for example, *Phaeodactylum tricorutum*), at least twice per week. Culture water should be aerated and renewed once a week.

Water

3. Natural or synthetic salt water may be used as the test medium. If natural seawater is used, it should be collected from an uncontaminated area known to support a healthy, reproducing population of *T. battagliai* or a comparable sensitive species. Natural salt water used for solutions dilution and in the toxicity test should be passed through a filter effective to ≤ 0.2 µm to remove suspended particles, organisms and facultative pathogens from the water. If synthetic salt water is used, it should be prepared by dissolving reagents of known analytical grade, or a commercially available formulation, in distilled or deionised water. Also, the water should be intensively aerated before use (≥ 90 % oxygen saturation), and aging for one to two weeks might be desirable.

Test solutions

4. Test solutions of the chosen concentrations are prepared by dilution of a stock solution in seawater. In the case of substance with low water solubility, stock solutions

may be prepared by ultrasonic dispersion or other suitable physical means. If necessary, vehicles (e.g. organic solvents) of low toxicity may be used, in concentrations that should not exceed 0.1 mL/L. When vehicles are used, an additional control to that of seawater should be exposed to the same concentration of the vehicle as that used in the most concentrated solution of the test substance.

Test procedure

Conditions of exposure

1. Duration: 48 h;
2. Photoperiod: 16:8 h light:dark
3. Temperature: 20 ± 1 °C;
4. Oxygen concentration: ≥ 60 % of the air saturation value;
5. Feeding: starving conditions;
6. Aeration: without aeration;
7. Water parameters: pH 8.0 - 8.3, salinity of ≈ 35 ‰; concentrations of both total organic carbon (TOC) and particulate matter should be less than 5 mg/L;
8. The dissolved oxygen, salinity and pH must be measured at the beginning and end of the test in the control(s) and the high, medium, and low concentrations of test treatments. In semi-static conditions measurement of these parameters should be performed in the renewal test solutions prior to the addition to the microwells. Measurement of ammonia, particulate matter, total dissolved gas, and TOC is desirable.

Note: The medium renewal and its frequency will depend on the stability of the test substance. There must be evidence that the concentration of the test substance being tested has been satisfactorily maintained (i.e. inside the range of 70 - 130 %). When the medium is renewed, a fixed volume (preferably 90-95 % of the total test medium volume) should be aspirated from each microwell chamber (e.g. with a syringe) under a stereomicroscope.

9. If the concentration of the test chemical is expected to remain within ± 20 % of the nominal values, it is recommended that, as a minimum, the controls (water and solvent if used), the highest and lowest test chemical concentrations, and the positive controls (if used) be analysed when freshly prepared at the start of the test and immediately prior to each renewal, for renewable tests. Additionally, if possible, some old removed/pooled test solutions (e.g. the highest and lowest test concentrations) should be measured. Used microwell seawater can be collected and pooled to yield 1-mL for chemical analysis from every 4-5 microwells within a given treatment. For tests where the concentration of the test chemical is not expected to remain within ± 20 % of the initial measured concentration it is necessary to analyse all control(s) and test concentrations when freshly prepared and at test medium renewal, for renewable tests.

Exposure

10. The nauplii should be exposed to at least five concentrations of the test chemical. Except for the control(s) (seawater and control solvent, if used) and the highest concentration, each concentration should be at least 60 % of the next higher one, unless information concerning the concentration-effect curve indicates that a different dilution

factor is more appropriate. If the estimate of acute toxicity is particularly uncertain six or seven concentrations might be desirable.

Note: The prediction of the concentrations to be tested might be based on the results of a test on the same or a similar chemical with the same or a similar species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test in which groups of five or more organisms are exposed for 24 to 96 h to a control and three to five concentrations of the test substance that differ by a factor of ten.

11. Nauplii are obtained by isolating ovigerous females from the stock culture (filtered through a 125 µm mesh sieve) in a crystallising dish containing 200 mL of seawater and $\approx 2 \times 10^{-5}$ cells/mL of algae. To limit the transfer of algal cells, nauplii are sieved carefully through a 62 µm nylon mesh and resuspended in freshly filtered seawater.

12. Four < 24 h-old nauplii are randomly selected, using a finely-drawn out Pasteur pipette under a stereomicroscope with dark-field illumination, and added to each well of a 24-well polystyrene plate, in order to keep a density of copepods less than 1 per 0.5 mL of solution (ISO, 1999). Marginal wells should be avoided and left empty of organisms. This will yield a maximum total initial test population of 160 individuals dispersed over 5 replicate plates. To reduce evaporation, wells along the margins as well as inter-well spaces of the microplate can be filled with Milli-Q water.

13. After all wells are successfully loaded with nauplius, the overlying transfer seawater is removed under a stereomicroscope using an analytical grade 500-µL glass syringe so that approximately 5 µL of seawater remains. This standardizes the starting volume in each well and allows for minimal dilution of the test solutions. Once the overlying water is removed, 2 mL of control(s) or test solution is added, aspirated, and added again to the appropriate wells using a graduated, glass capillary pipette.

14. The microplates should be covered and placed in a clean, temperature-regulated incubator or room to keep out extraneous contaminants and to reduce evaporation of test solution. Care has to be taken so that the evaporation losses do not exceed 10 % on a daily basis. To prevent such a scenario, microplates can be placed in a 0.2-0.3 m³ humidity box on a platform over a water-filled or water-saturated sponge bath inside the temperature regulated incubator or room. Microplates should be randomly allocated and handled.

Observations

15. Animal mortality is observed and recorded at 24 and 48 h. The criteria for dead copepods are opaque white coloration, immobility, and lack of reaction to gentle tapping of the microplate.

16. If it can be done without stressing live organisms, dead organisms should be removed at least once every 24 h. All organisms used in the test should be destroyed at the end of the test.

Reference test

17. A reference chemical (e.g. zinc sulphate or copper sulphate) may be tested in parallel periodically as a means of assuring that the test protocol and test conditions are reliable, and that the sensitivity of copepod test stocks is consistent. LC50 results for

reference tests should fall within acceptable limits as set by internal culture control charts or in the literature.

Results

1. The cumulative percentage mortality for each exposure period is plotted against concentration on a logarithmic scale;
2. The 48 h LC50 is calculated by normal statistical procedures, if possible (confidence limits ($p=0.95$)).

Validity of the test

1. The average mortality in the control(s) should not exceed 10 % at the end of the exposure period;
2. Calculation of the LC50 should usually be considered unacceptable if either or both of the following occurred:
 - a. No treatment other than a control treatment killed or affected less than 37 % of the test organisms exposed to it.
 - b. No treatment killed or affected more than 63 % of the organisms exposed to it.

References

- ASTM E 2317-04, 2004. Standard guide for conducting renewal microplate-based life-cycle toxicity tests with a marine meiobenthic copepod. American Society for Testing and Materials, West Conshohocken, PA.
- ASTM E 729-96 (2002), 2003. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials, West Conshohocken, PA.
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- Williams, T.D., Jones, M.B., 1994. Effects of temperature and food quantity on postembryonic development of *Tisbe battagliai* (Copepoda: Harpacticoida). *J Exp Mar Biol Ecol.* 183: 283-298.

2.1.1.3. Mysid acute toxicity test

Based on EPA OPPTS 850.1035.

Principle of the test

This guideline prescribes a test using mysids as test organisms to develop data on the acute toxicity of chemicals. In preparation for the test, test chambers are filled with appropriate volumes of dilution water and the test chemical is added to them. The test is started by randomly introducing mysids into the test chambers, which are observed periodically during the test. Dead mysids are removed, and the findings recorded. The data collected during the test are used to develop concentration-response curves and the 48- and 96-h LC50 values for the test substance. These data can be used in assessing the hazard of a chemical to the aquatic environment.

Test conditions

Equipment

- Flow-through or recirculating tanks for holding and acclimating mysids;
- Mechanism for maintaining the water temperature during the holding, acclimation, and test periods;
- Thermometers;
- Apparatus for straining particulate matter, removing gas bubbles, or aerating the water, as necessary;
- Apparatus for providing adequate photoperiod;
- For flow-through tests, flow-through chambers and a test substance delivery system;
- For static tests, suitable chambers for exposing test mysids to the test substance are required.

Note: Materials and equipment that contact test solutions should be chosen to minimize sorption and leach of test chemicals from and into the dilution water in quantities that can affect test results.

Species

1. The mysid, *Americamysis bahia*, is the organism specified for these tests. Either juvenile (< 24 h old) or young adult (5-6 days old) can be used, and should originate from laboratory cultures in order to ensure the individuals are of similar age and experimental history. Mysids used for establishing laboratory cultures may be purchased commercially or collected from appropriate natural areas. Mysids used in a particular test should exhibit normal behaviour, be of similar age and be of normal size and appearance for their age. Also, they should not have been used in a previous test, either in a control or a treatment group.

Acclimatisation

2. During acclimation mysids should be maintained in facilities with background colours and light intensities similar to those of the testing areas. Also, mysids should be cultured and tested in dilution water from the same origin. Any change in the

temperature and chemistry of the dilution water used for holding or culturing the test organisms to those of the test should be gradual. Within a 24-h period, changes in water temperature should not exceed 1 °C, while salinity changes should not exceed 5 %. Any food utilized should support survival, growth, and reproduction of the mysids. A recommended food is live *Artemia* spp. (48-h-old nauplii). Methods for the care and handling of mysids during holding, culturing and testing periods can be consulted in EPA, 1978.

Water

3. Either natural or artificial seawater can be used as dilution water. Natural seawater should be filtered through a filter with a pore size of < 20 µm prior to use in a test. Artificial seawater can be prepared by adding commercially available formulations or specific amounts of chemicals of known analytical grade to deionized water. Deionized water with a conductivity less than 1 µohm/cm at 12 °C is acceptable for making artificial seawater.

Test solutions

4. Deionized water should be used in making stock solutions of the test substance. Test solutions of the chosen concentrations are prepared by dilution of a stock solution in seawater. If necessary, solvents of low toxicity may be used, in concentrations that should not exceed 0.1 mL/L. Preferred carriers are dimethyl formamide, triethylene glycol, acetone, or ethanol. In these instances, an additional control to that of seawater should be exposed to the same concentration of the vehicle as that used in the most concentrated solution of the test substance.

Test procedure

Conditions of exposure

1. Duration: 96 h;
2. Photoperiod: 14:10 h light:dark photoperiod with a 15 to 30 min transition period;
3. Temperature: 25 ± 2 °C;
4. Oxygen concentration: 60 - 105 % of the air saturation value;
5. Feeding: daily (e.g. live *Artemia* spp., 48-h-old nauplii);
6. Aeration: with aeration;
7. Water parameters: pH 8.0 - 8.3, salinity 20 ± 3 ‰;
8. If a flow-through test is performed, the water flow through each chamber is adjusted to the rate desired. In addition, the rate at which the test substance is added is adjusted to establish and maintain the desired concentration of test substance in each test chamber. The 24-h flow through a test chamber should be equal to at least 5× the volume of the test chamber;
9. The dissolved oxygen concentration, temperature, salinity, and pH should be measured at the beginning and end of the test in each chamber. The system to be used in flow-through tests should be calibrated before each test, determining the flow rate through each chamber and the concentration of the test substance in each chamber. The general operation of the test substance delivery system should be checked twice daily during a test. The concentration of the test substance in the chambers should be measured as often as is feasible during the test. During static tests, the concentration of

test substance should be measured at a minimum at the beginning and at the end of the tests. During the flow-through test, the concentration of test substance should be measured at the beginning and end of the test and in at least one appropriate chamber whenever a malfunction is detected in any part of the test substance delivery system. Equal aliquots of test solution may be removed from each replicate chamber and pooled for analysis.

Range-finding test

10. A range-finding test should be conducted to determine which life stage and test solution concentrations are to be utilized in the definitive test. The mysids should be exposed to a series of widely spaced concentrations of test substance (e.g. by a factor of 10), usually under static conditions. This test should be conducted with both newly hatched juvenile (< 24 h old) and young adult (5 to 6 days old) mysids. For each age class a minimum of 10 mysids should be exposed to each concentration of test chemical for up to 96 h. The age class which is most sensitive to the test substance should be utilized in the definitive test. When no apparent difference in sensitivity of the two life stages is found, juveniles should be utilized. No replicates are required, and nominal concentrations of the test chemical are acceptable.

Exposure

11. The test should be conducted on the mysid life stage (juveniles or young adults) which is most sensitive to the test substance being evaluated.

12. A minimum of 20 mysids per concentration should be exposed to 5 or more concentrations of the test chemical chosen in a geometric series in which the ratio is between 1.5 and 2.0. An equal number of mysids are introduced into the test and control(s) (control seawater and solvent, if used) chambers, with 2 or more replicates, by stratified random assignment. In addition, test chambers within the testing area should be positioned in a random manner or in a way in which appropriate statistical analyses can be used to determine the variation due to placement.

13. It is recommended that mysids be held in retention chambers (constructed with netting material of appropriate mesh size) within test chambers to facilitate observations and eliminate loss of test organisms through outflow water.

14. Test chambers should be loosely covered to reduce the loss of test solution or seawater due to evaporation and to minimize the entry of dust or other particulates into the solutions.

Observations

15. Each test chamber should be checked for dead mysids at 24, 48, 72, and 96 h after the beginning of the test. Dead mysids should be counted and the number registered. In addition to death, any abnormal behaviour or appearance should also be reported.

Results

1. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves at all time-point observations.

2. A 48- and 96-h LC50 values should be determined along with their 95 % confidence limits.

3. An NOEC and the slope of the dose-response curve should also be determined.

Validity of the test

1. The average mortality or abnormal behaviour in the control(s) should not exceed 10 % at the end of the 96 h exposure period.
2. The measured concentration of the test substance should not vary more than 20 %.
3. During a test, the flow rates should not vary more than 10 % among test chambers or across time.

References

U.S. Environmental Protection Agency, Bioassay Procedures for the Ocean Disposal Permit Program, EPA Report No. 600-9-78-010 (Gulf Breeze, Florida, 1978).

U.S. Environmental Protection Agency, 1996. Ecological Effects Test Guidelines – OPPTS 850.1035 Mysid Acute Toxicity Test. EPA 712-C-96-136.

2.1.1.4. Marine algal growth inhibition test

Based on EPA OPPTS 850.54400 and ASTM E 1218-04.

Principle of the test

This guideline covers procedures to develop data on the phytotoxicity of chemicals added to nutrient growth medium on growth of a saltwater algae (*Skelatonema costatum*) for a period of 96 hours (h), under static exposure. At the end of 96 h, and at the end of 24, 48, and 72 h if possible, enumeration of the algal cells in all containers enables the determination of growth inhibition or stimulation. These data are used to define the concentration-response curve and calculate the EC50 value (chemical concentration to effect 50 % of algal growth) at these times.

Although the test duration is comparable to an acute toxicity test with aquatic animals, an algal toxicity test of short duration allows for examination of the effects upon multiple generations of an algal population and thus should not be viewed as an acute toxicity test.

Note: Static tests might not be applicable to chemicals that are highly volatile, are rapidly biologically or chemically transformed in aqueous solutions, or are removed from test solutions in substantial quantities by the test vessels or organisms during the test.

Test conditions

Equipment

- Controlled environment room or growth chamber in which a constant temperature and uniform illumination can be maintained;

- Erlenmeyer flasks of any volume between 125 and 500 mL as long as the same size is used throughout a test and the test solution volume does not exceed 50 % of the flask volume;
- Stainless Steel Caps, Shimatsu Enclosures, Foam Plugs, Glass Caps, or Standard Screw Caps, (plastic/bakelite);
- pH Meter;
- Calibrated Light Meter;
- Pipettes;
- Rotary shaker;
- Centrifuge and centrifuge tubes;
- Filtration apparatus and membrane filters with 0.22- μm pore size;
- Apparatus for determining cell concentrations (e.g. microscope, capable of 100 to 400 \times magnification, with counting chamber);
- Autoclave or microwaving.

Note: test vessels and equipment used to prepare and store growth medium, stock solutions, and test solutions should be cleaned and sterilized before use. New items should be washed with detergent and rinsed with water, a water-miscible organic solvent, water, acid (such as 10 % concentrated hydrochloric acid), and at least twice with deionized or distilled water. Glassware should be sterilized by autoclaving for 20 min at 121°C and 1.1 kg/cm² or by microwaving.

Species

1. The species most widely used for testing in saltwater is the diatom *Skeletonema costatum*. Other species that have been successfully used are the centric diatom *Thalassiosira pseudonana*, the flagellate *Dunaliella tertiolecta* and the golden-brown alga *Phaeodactylum tricorutum*. Because the sensitivities of algal species often differ substantially, it is usually desirable to conduct tests with two or more species from different phyla.

Acclimatisation

2. Algae to be used in acute toxicity tests may be initially obtained from commercial sources and subsequently cultured using sterile technique. Sterile Erlenmeyer flasks of borosilicate glass or polycarbonate are usually used as test and culture vessels. Stock algal cultures should be shaken on a rotary shaking apparatus. Toxicity testing should not be performed until algal cultures (from the same source and the same stock culture) are in a logarithmic growth phase in at least two subcultures lasting 7 days each prior to the start of the test.

Growth medium water (Annexe 1)

3. Growth medium water for tests with saltwater algae is prepared by adding appropriate amounts of specified chemicals to (0.22- μm membrane filter) natural seawater or artificial saltwater (prepared by adding a commercial synthetic sea salt formulation or a modified synthetic seawater formulation to distilled/deionized water). Salinity should be of 30 ‰ (24 to 35 g/kg), and may be adjusted by adding ASTM Type I water, NaCl, or sea salts. It may be desirable to reduce the amount or omit EDTA from the growth medium in toxicity testing if it is suspected that the chelator will interact with the test

chemical. The pH of the growth medium is to be 8.1 (± 0.1) at the start of the test and may be adjusted prior to test chemical addition with 0.1N or 1N NaOH or HCl. The pH is not adjusted after the addition of the algae.

Test solutions

4. In some cases the test chemical can be added directly to the growth medium, but usually it is dissolved in a solvent to form a stock solution that is then added to the growth medium. If an organic solvent is used to dissolve the chemical, its volume should be kept to a minimum. The upper limit of solvent volume should not exceed 0.5 mL/L, preferably 0.1 mL/L, and the same amount of solvent should be added to each test concentration. For some chemicals, the stock solution must be prepared with dilution water (e.g. ASTM Type I water). Volumetric addition should be limited to prevent dilution of nutrients in the growth medium ($< 10\%$ of the total volume).

5. If a solvent is used, one solvent control containing the same concentration as the used in the test solutions must be included in the test in addition to the growth medium control.

Test procedure

Conditions of exposure

1. Duration: 96 h;
2. Photoperiod: 14:10 h light:dark;
3. Light: Fluorescent lights providing 4.3 K lx ($4,306 \text{ lm/m}^2$ or $400 \pm 10\%$ fc) are to be used. These lamps should have a photosynthetically active radiation of approximately $66.5 \pm 10\%$ mEin/m²/sec;
4. Temperature: $20 \pm 2^\circ\text{C}$;
5. Water parameters: pH 8.0 ± 0.1 , salinity of 30 ‰;
6. Rate of oscillation: 60 cycles/min;
7. Temperature should be recorded hourly during the test, with a continuous recording device. Light intensity should be monitored at the beginning of the test at each test vessel position and should not differ by more than 15% from the selected intensity. The pH of all test solutions is to be measured at the beginning and end of the test. If the test chemical is highly acidic and reduces the pH of the test solution below 5.0 at the first measurement, appropriate adjustments to pH should be considered, and the test solution measured for pH on each day of the test. The rate of oscillation should be determined at the beginning of the test or at least once daily during testing if the shaking rate is changed or changes. The concentration of the test chemical in the test solutions at the beginning and end of the test are to be determined. At the end of the test the replicate test vessels for each chemical concentration may be pooled into one sample. An aliquot of the pooled sample may then be taken and the concentration of test chemical determined after all algal cells have been removed. In addition, the concentration of test chemical associated with the algae alone may be determined. Separate and concentrate the algal cells from the test solution by centrifuging or filtering the remaining pooled sample and measure the test substance concentration in the algal-cell concentrate.

Exposure

8. Algae should be exposed to five or more concentrations of the test chemical in a geometric series in which the ratio is between 1.5 and 2.0. Often it is possible to choose test chemical concentrations based on the anticipated slope of the concentration-response curve, and these concentrations should result in greater than 90 % of algal growth being inhibited or stimulated at the highest concentrations of test substance compared to control(s) or that the test concentrations should bracket the expected EC50 value. Algae are to be placed in a minimum of three replicate test vessels for each concentration of test chemical and ideally twice that number of control(s) (nutrient growth medium and, if used, solvent control).

Note: The prediction of the EC50 might be based on the results of a test on the same or a similar test chemical with the same or a similar species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test in which the test species is exposed to a control and three to five concentrations of the test chemical that differ by a factor of ten.

9. The test begins when algae (inocula) from 3 to 7 day-old stock cultures are placed in the test vessels containing equal volumes of test solution, having the appropriate concentrations of the test substance. At the onset of testing, each test vessel should be inoculated at an initial cell concentration of approximately 7.7×10^4 cells/mL of test solution.

10. Closures of test vessels must be loose-fitting or porous, and they should be placed on a rotary shaking apparatus and oscillated at approximately 60 cycles/min during the test. If clumping of *Skeletonema* is experienced or anticipated, hand shaking once or twice a day is acceptable. Test vessels should be randomly assigned in a growth chamber or controlled environment room.

11. Algal growth in controls should reach the logarithmic growth phase by 96 h, at which time the concentration of *Skelatonema* should be approximately 1.5×10^6 cells/mL. If logarithmic growth cannot be demonstrated, the test is to be repeated.

Observations

12. At the end of 96 h, and, if possible, at the end of 24, 48, and 72 h, the algal growth response (number or weight of algal cells per millilitre) in all test vessels and control(s) is to be determined by an indirect (spectrophotometry, electronic cell counters, dry weight, etc.) or a direct (actual microscopic cell count of at least 400 cells per flask; two samples should be taken from each test vessel and two counts made of each sample) method. Indirect methods are to be calibrated by a direct microscopic count or data should be presented that relate electronic counts with microscopic counts. The percentage inhibition or stimulation of growth for each concentration, EC50, and the concentration-response curves are determined from these counts.

13. At the end of the definitive test, the following additional analyses of algal growth response are to be performed:

- a. Determine whether the altered growth response between controls and test algae (in the highest test chemical concentrations) was due to a change in relative cell numbers, sizes, or both. Also note any unusual cell shapes, colour differences, differences in chloroplast morphology, flocculation, adherence of algae to test

vessels, or aggregation of algal cells. These observations are qualitative and descriptive, and are not used in end-point calculations. They can be useful in determining additional effects of tested chemicals.

- b. In test concentrations where growth is maximally inhibited, algistatic effects may be differentiated from algicidal effects by a recovery phase. An aliquot of 0.5 mL from each of the replicate test vessels of the highest test concentration causing algal growth inhibition is removed and combined in a single clean flask containing sufficient fresh growth medium to dilute the test chemical to an insignificant concentration. Aliquots from the control(s) vessels are also transferred to clean medium. Incubate these subcultures under the same conditions used for the exposure phase for a period of up to 9 days, and observe periodically for algal growth (direct or indirect methods) to determine if the algistatic effect noted after the 96 h test is reversible. This subculture test may be discontinued as soon as growth occurs.

Reference test

14. Positive controls using zinc chloride as a reference chemical should also be run periodically to determine if the test algae are responding to a known chemical in the expected manner, ensuring that stock and test conditions are reliable. At least three concentrations of the reference chemical are run at or near the expected median effect level.

Results

1. The growth rate for each test vessel over the selected time interval is calculated as follows:

$$u = (\ln N2 / \ln N1) / t$$

where:

u = growth rate,

$N1$ = initial cell concentration expressed as cells/mL,

$N2$ = final cell concentration expressed as cells/mL,

t = elapsed time between the measurements, in days.

Where the time interval is the entire test (96 h), the resulting growth rate is the average specific growth rate.

2. The mean percent inhibition at each test concentration, is calculated as follows:

$$\% I = (C - X) / C * 100$$

where:

C = the average growth rate for the control test vessels, and

X = the average growth rate for an individual test treatment vessel or the average value for the treatment.

Mean and standard deviation should be calculated and plotted for each treatment and control.

3. The EC50 value (96 h) and associated 95 % confidence limits are determined using appropriate statistical methodology. Statistical analysis should provide a goodness-of-fit determination for the concentration-response curves, which are plotted using the mean measured test solution concentrations obtained in the test vessels at the end of the test.
4. Results from the recovery phase are used to determine the algistatic concentration;
5. It might be desirable to perform a hypothesis test to determine which of the tested concentrations of test chemical caused a statistically significant inhibition in growth. Presentation of results of each hypothesis test should include the test statistic and its corresponding significance level, the minimum detectable difference, and the power of the test.

Validity of the test

Cell counts in the controls must increase by a factor of at least 16 during a 72-h period (corresponding to a specific growth rate of 0.9 day^{-1} and the coefficient of variation in the controls must be inferior to 7 %.

References

- ASTM E 1218-04, 2004. Standard guide for conducting static toxicity tests with microalgae. American Society for Testing and Materials, West Conshohocken, PA, 19428-2959, United States.
- U.S. Environmental Protection Agency, 1996. Ecological Effects Test Guidelines – OPPTS 850.54400 Algal Toxicity, Tiers I and II. EPA 712-C-96-164.
- Walsh, G.E., Alexander, S.V., 1980. A Marine Algal Bioassay Method: Results with Pesticides and Industrial Wastes. *Water Air Soil Pollut.* 13(1): 45-55.

Annexe 1. Preparation of growth saltwater medium (ASTM, 2004; Walsh and Alexander, 1980)

Table 1. Chemical composition of growth medium.

Metal mix (A)	
FeCl ₃ ·H ₂ O	0.048 g
MnCl ₂ ·4H ₂ O	0.144 g
ZnSO ₄ ·7H ₂ O	0.045 g
CuSO ₄ ·5H ₂ O	0.157 mg
CoCl ₂ ·6H ₂ O	0.404 mg
H ₃ BO ₃	1.140 g
Na ₂ EDTA·2H ₂ O	1.0 g
Minor Salt Mix (B)	
K ₃ PO ₄	0.3 g
NaNO ₃	5.0 g
NaSiO ₃ ·9H ₂ O	2.0 g
Vitamin Stock Solution (C)	
Thiamine Hydrochloride	500 mg
Biotin	1 mg
B12	1.0 mg

1. Prepare Stock Solution A by adding the specific amount of chemicals in the order listed in the table to 900 mL ASTM Type I water and dilute to 1 L.
2. Prepare stock solution B by adding the specific amounts of the chemicals listed in the table to 900 mL ASTM Type I water and dilute to 1 L.
3. Prepare Stock Solution C by adding the specified amount of chemicals in the order listed in the table to 900 mL ASTM Type I water and dilute to 1 L.
4. The stock solutions are added to a sterile container containing either natural salt water that has been filtered through a 0.22 µm membrane filter or reconstituted salt water. Add the amounts given in the table to prepare medium used for toxicity testing. Add twice the amounts given to prepare medium for use in maintenance of stock cultures.
5. Add 15 mL of metal Stock Solution (A)/L of medium.
6. Add 10 mL of minor salt Stock Solution (B)/L of medium.
7. Add 0.5 mL of vitamin Stock Solution (C)/L of
8. Adjust pH to 8.0 ± 0.1 with 0.1 N or 1.0 N NaOH or HCl.
9. Store medium in the dark at approximately 4 °C until use.

2.1.1.5. Oyster embryo-larval bioassay

Based on ICES Techniques in Marine Environmental Sciences no. 54.

Principle of the test

This guideline covers procedures to assess toxicity effects of aqueous environmental samples on embryo-larval development of the Pacific oyster (*Crassostrea gigas*), under static exposure. At the end of 24 hours (h) of exposure, the ability of early stage embryos to develop normally and reach the “D-shaped” larval stage is assessed. Although the exposure time is short, it encompasses a period of intense cellular activity during which the impairment of a number of critical physiological and biochemical processes may result in poor and abnormal growth and development. The endpoint values for an oyster embryo-larval development toxicity test are based on the percentage of abnormal embryos in each test concentration (e.g. 24 h-EC50, the effective concentration that results in 50 % of the exposed oysters’ embryos failing to reach D-shaped larvae).

Test conditions

Equipment

- Test vessels (stoppered vials capable of holding 30 mL of test solution or multi-well plates capable of holding 5 mL of test solution) made of non-toxic inert material (e.g. glass or polystyrene);
- A constant temperature room;
- A microscope (inverted or binocular) providing 20 - 100 x magnification and a grid marked counting chamber (e.g. 1 mL Sedgewick-Rafter cell);
- Equipment for measuring pH, dissolved oxygen, salinity, temperature and other parameters as appropriate;
- Recording equipment for counting egg and larval numbers;
- Pasteur pipettes;
- A large flat-bladed scalpel or oyster knife;
- Glass beakers;
- Plastic meshes (60 and 100 µm).

Species

1. Oysters (*C. gigas*) can be conditioned in-house or purchased from recognised commercial sources, in which case they should be delivered to the laboratory within 24 h of dispatch.

Acclimatisation

2. Oysters should normally be used within a few hours of being received in the laboratory and require no maintenance. However, if necessary, the oysters can be kept overnight provided they are stored in air under damp (e.g. wrapped in paper moistened with seawater placed in a box) and refrigerated (6 - 10 °C) conditions. If oysters are held in this manner they should be allowed to recover before being used, by placing them in individual beakers of aerated seawater (1 L) for about 1 h at 20 - 22 °C. Oysters kept

under these conditions may spawn naturally and the resulting gametes can be used in the test. If prolonged storage is necessary, the oysters may be placed in aerated sea water at $< 12\text{ }^{\circ}\text{C}$ and provided with food (suspension of algae). This way the oysters may remain in good condition for at least two or three days.

Water

3. Natural or artificial seawater may be used. Natural seawater should be obtained from a reference site, known to be free from significant contamination. An assessment of the natural seawater quality should be carried out, by monitoring parameters that are known to be toxic to aquatic organisms and the respective concentrations should be lower to those deemed safe for oyster embryo development. Artificial seawater may be prepared by adjusting distilled or deionised water to a salinity of $34 \pm 2\text{ }_{\text{‰}}$ with analytical grade sea salts. This should be aged by at least 24 h prior to use and undergo continual aeration between preparation and use.

Test solutions

4. Test solutions for the determination of the toxicity of environmental samples, can be used either as received or using a range of concentrations. The dilution water used for controls and dilution of samples should be a reference seawater, free from significant contamination.

5. When a range of concentrations is used, an appropriate series of concentrations should be prepared with a factor not exceeding 2.2. See Table Y for the preparation of typical test solutions comprising 200 mL of test solution.

6. On the day the toxicity test is to be carried out, the concentration range should be prepared in volumetric flasks by diluting (with reference seawater) appropriate amounts of the environmental contaminated seawater. If appropriate, test dilutions can be prepared directly in the test vessels. At least four replicate test vessels should be used for each test concentration, along with six replicates of the control. The remaining test solution should be used to determine the selected water quality parameters.

7. The approach taken for samples where any of the threshold criteria for the test solutions fall outside of the limits specified for the physico-chemical parameters adequate for oyster's development should be adjusted. This should, wherever possible, be restricted to specific test dilutions rather than to the whole sample and, if possible, both adjusted and unadjusted dilutions should be tested concurrently.

8. A buffered formaldehyde solution will be needed. Dissolve 5 g of sodium tetraborate in 250 mL of distilled or deionized water. Add this solution to 250 mL of 40 % v/v formaldehyde solution. The combined mixture may be stored in a screw top glass bottle. This solution may be freshly prepared or stored for up to six months at $4\text{ }^{\circ}\text{C}$.

Table 2. Preparation of test dilutions.

Nominal concentration (%)	Volume of reference seawater (mL)	Volume of contaminated seawater (mL)
0 (control solution)	200	0
0.1	199.8	0.2
0.22	199.5	0.44
0.46	199.1	0.92
1.0	198.0	2.0
2.2	195.6	4.4
4.6	190.8	9.2
10.0	180	20
22.0	156	44
46.0	108	92
100	0	200

Test procedure

Conditions of exposure

1. Duration: 24 ± 2 h;
2. Photoperiod: without light;
3. Temperature: $24 \pm 2^\circ\text{C}$;
4. Oxygen concentration: $\geq 60\%$ of the air saturation value.
5. Water parameters: pH 6.0 – 8.5 at the start of the test, salinity of 22 - 36 ‰;
6. At the beginning and end of the test period, the determination of pH, dissolved oxygen, temperature and salinity should be made on the test solutions specifically reserved for monitoring water quality.

Test preparation

7. The embryos are produced from the sperm and eggs of conditioned adult male and female oysters, each of individual whole wet weight > 45 g. Male and female gametes are obtained by stripping the gonads or by naturally spawning the adults (e.g. temperature shock). Irrespective of the approach used to strip the gametes, it is essential that all the gametes be collected at the same time.
8. If stripping the gametes, the shell of the mature conditioned oysters should be scrub to remove adhering debris and then the oysters opened, by breaking the hinge and cutting the adductor muscle with a standard oyster knife. The knife should be inserted in the flat edge of the oyster and held level when cutting to avoid damaging the gonads. The body cavity of each oyster should be thoroughly rinsed with seawater to remove any debris which may be present. Additionally, prior to stripping sperm from a male oyster, a small sample of sperm from each male should be transferred to a slide together with a few drops of seawater and, after 15 – 30 min, observed under a microscope (at 100 x) to assess the activity of the sperm. Male oysters with the most motile sperm are selected for stripping.
9. The gametes may be stripped by one of the two methods:

- a. A clean Pasteur pipette may be inserted into the gonad to a depth of 1 - 2 mm and the eggs or sperm collected. The gametes should be transferred to separate volumes of seawater and held at 24 ± 2 °C.
 - b. The gonad may be gently cut into with a sharp scalpel, angling the blade upwards to avoid puncturing the gut as contamination of gametes can lead to reduced fertilization. Gametes should be collected, by pipetting seawater (at 24 ± 2 °C) over the surface of the gonad and washing the gametes into a suitable vessel.
10. The sperm suspensions from male oysters should be filtered through a 60 µm mesh sieve and collected in a clean glass beaker which removes any tissue debris. The filtered sperm suspensions should then be mixed in a clean glass beaker.
11. The suspension of eggs from each female oyster should be filtered through a 90 - 100 µm plastic mesh sieve, and collected in a clean glass beaker. This should remove any tissue debris. Individual filtered egg suspensions can then be stored, to ensure selected eggs are obtained from the ripest gonads, or mixed egg suspensions can be prepared in a single beaker from oysters that all possess ripe gonads. An aliquot of the individual or mixed suspensions (1 – 5 mL) should then be removed and examined microscopically using an appropriate chamber to assess the quality and egg population densities (number of eggs/mL). Batches of eggs with high proportions of abnormal eggs (e.g. those that fail to ‘round up’ after exposure to seawater) should be discarded. Egg population densities may also be assessed using a Coulter counter. The egg suspension should then be adjusted with an appropriate volume of seawater to obtain an egg density of approximately 3000 - 6000 eggs/mL and be gently mixed every 5 min.
12. Fertilization should be carried within 30 minutes of obtaining the egg and sperm suspensions by fertilizing the egg suspension from each female with the combined sperm suspension and subsequently combining individual viable and healthy embryo suspensions, or by fertilizing the combined egg suspension with the combined sperm suspension. The volume ratio should be 2 - 3 mL of sperm suspension to 1-L of individual/mixed egg suspension. After mixing the suspensions, the eggs should be checked within 15- 30 minutes to ensure fertilization is occurring. By this time, sperm should surround each egg and polar bodies should be evident.
13. The embryo suspension should then be incubated for 2 h at 24 ± 2 °C in the dark, without aeration. The suspension should be stirred at least every 10 - 15 minutes to prevent excessive settling of embryos and potential oxygen starvation. After approximately 2 h, the embryos should be assessed microscopically (at 20 - 40 x) using an appropriate counting chamber to determine whether cell cleavage is occurring. During this 2-h period, the eggs should undergo the early stages of cleavage and typically reach the 16 - 32 cell stage. If the embryos have not yet reached these stages, the embryo suspension may be left for up to an additional 2 h to allow this level of development to be attained. If, after this time, this level of development has not been reached, the preparation should be discontinued and other oysters stripped for gametes.

Exposure

14. 100 embryos/mL per test vessel should be exposed to a series of concentrations and to controls (reference seawater). Typically, a volume of approximately 20 µl of embryo

suspension per mL of test solution is required. The embryo suspension should be vigorously mixed between each transfer to maximise homogeneity in transfers between each test vessel.

Note: the number of inoculated embryos in each test vessel may be checked using the egg count check procedure. For this procedure, three additional control replicates should be inoculated and then immediately fixed, for posterior counting to provide an estimate of the total numbers of larvae supplied to each test vessel.

15. The inoculated test dilutions should be incubated at 24 ± 2 °C under static conditions without light.

16. The test should be terminated after 24 ± 2 h. Buffered formaldehyde solution should be added to each test vessel (20 µl of buffered formaldehyde solution per mL of test dilution), in a fume cupboard, to fix and preserve the larvae.

Observations

17. The number of normal D-shaped larvae is counted microscopically using a suitable counting chamber. In the context of these procedures, normal development describes the transformation of embryos into “D-shaped” larvae having a protective D-shaped shell where the paired hinged shells are visible. The bivalve shell may be irregular to some degree.

18. The number of abnormal embryos/larvae is also to be counted and is defined as those embryos which die at an early stage or larvae which develop but which fail to reach the D-shaped stage, although they may be normal trochophores, or other early larval stages. In some cases, larvae may completely disintegrate under toxic challenge and it may not be possible to accurately determine the numbers that are abnormal. Unfertilized eggs should not be included in the count, as they do not represent viable test organisms undergoing development.

Note: Care should be taken when aliquots of the test dilutions are taken for counting to ensure they are representative of the test replicate as a whole. This can be achieved by thorough mixing of the test dilution immediately prior to sub-sampling.

19. Digital imaging systems may be used as an alternative to conventional assessment, ensuring, however, previous validation before use.

Reference test

20. Positive controls using zinc (as zinc sulphate) as a reference chemical should also be run periodically, according to the procedures described above.

Results

1. The number of abnormal embryos is calculated to be 100 minus the number of normal D-shaped larvae. The additional abnormalities in the test samples, compared with those in the control, is expressed as the Percent Net Response (PNR):

$$\text{PNR} = \frac{\% \text{ test abnormality} - \% \text{ control abnormality}}{100 - \% \text{ control abnormality}} \times 100$$

The calculation of the results is based on the assumption that there were 100 embryos exposed in each 2 ml of test or control sample. This assumption may not be valid because of procedure errors¹.

2. The data (i.e. percentage of normal D-shaped and abnormal larvae observed) should be used to determine:

- a. The 24 h- EC10, EC20 EC50 values, the effective concentrations that results in 10 %, 20 % and 50 % of the exposed oyster embryos failing to develop into normal D-shaped larvae after 24 h, respectively;
- b. The 24 h-NOEC value, the highest no-observed effect concentration after 24 h;
- c. The 24 h-LOEC value, the lowest observed effect concentration after 24 h.

3. The EC values should be determined using appropriate statistical procedures. Confidence limits (p=0.95) for the calculated EC value should be determined and quoted with the test results.

4. The NOEC and LOEC values should be determined using hypothesis testing.

The data derived, as explained in this guidance, should be arc sine square root transformed prior to statistical treatment using analysis of variance.

Note 1: when analysing data from the oyster embryo-larval development test several factors should be considered which will affect the choice of the different appropriate statistical procedures. For detailed information consult Leverett and Thain, 2013

¹Additional procedures can be introduced into the method which would help to achieve the nominal egg density. In general, the mean ‘egg count check’ value or mean control total value (i.e. normal D-shaped and abnormal) counts for the controls may be used to provide an estimate of total numbers of larvae in the test vessels, because neither of these values will be affected by potential larval disintegration. However, this means that an estimated total value is used for all test vessels (unlike the more direct procedure described above where all test vessels undergo specific counting of normal and abnormal larvae). In these situations, further manipulation of the data is required before they can be used to estimate the test endpoints.

This includes calculating for each test vessel, the percentage normal development (PND) and percentage abnormal development (PAD) values, the total of which is by definition 100 % (PND + PAD = 100).

The PND value depends on the manner by which the mean values of estimating the total numbers of larvae in each test vessel are estimated.

If the egg count check procedure is used, then

$$\text{PND} = \frac{\text{Number of normal D-shaped larvae}}{\text{Mean number of embryos from egg count check}} \times 100$$

If counts from controls are used, then

$$\text{PND} = \frac{\text{Number of normal D-shaped larvae}}{\text{Mean total number of normal D-shaped + abnormal embryos in controls}} \times 100$$

The percentage abnormal development (PAD) value is then calculated by subtracting the PND value from 100 ($PAD = 100 - PND$).

The mean PND_m and PAD_m values for each test concentration should then be determined from the replicate values.

For each test concentration, the percentage net response (PNR) value, which is effectively the response adjusted for the control (background) levels of abnormality, should then be calculated. Hence

$$PNR_{(TC)} = [(PAD_{m(TC)} - PAD_{m(C)}) / (100 - PAD_{m(C)})] \times 100$$

Where $PAD_{m(TC)}$ is the mean PAD value for the test concentration and $PAD_{m(C)}$ is the mean PAD value for the control.

Test endpoints can then be estimated using the $PNR_{(TC)}$ values for each test concentration.

Validity of the test

The results of the toxicity test should be rejected if the percentage of abnormal larvae in the controls is greater than 40 %.

References

Leverett, D., Thain, J., 2013. Oyster embryo-larval bioassay (revised). ICES Techniques in Marine Environmental Sciences no. 54. 34 pp.

2.1.1.6. Sea urchin embryo bioassay

Principle of the test

This guideline prescribes a test using an echinoderm as model organism to develop data on the acute toxicity of chemicals. Because of its abundance and broad geographical distribution in European waters, the sea urchin, *Paracentrotus lividus*, is recommended as test species. Alternatively, the proposed method is directly applied to other echinoid species used in ecotoxicology worldwide, such as the *Strongylocentrotus* and *Arbacia* genus. Embryos are exposed in well plates to seawater (*i.e.*, control) and to the test chemical at a range of concentrations, during 48 hours (h), under static conditions. Two toxicity criteria are applied as endpoints: size increase (quantitative) and morphological abnormalities (qualitative).

Test conditions

Equipment

- Controlled environment room or incubation chamber in which a constant temperature can be maintained;

- 3 mL microwell plates;
- Glass Pasteur pipettes;
- 5 mL plastic vials;
- 100 mL measuring cylinder;
- Plastic plunger;
- Inverted microscope with a graduated ocular or image analysis system;
- Counting chamber (1 mL Sedgewick-Rafter cell);
- Knife;
- Safety gloves;
- Equipment for measuring pH, dissolved oxygen, salinity, temperature and other parameters as appropriate;
- 40 % formaldehyde.

Species

1. Sea urchin (*Paracentrotus lividus*) is an herbivorous echinoderm present on rocky bottoms from intertidal and subtidal zones. Sea urchin high sensitivity, easy fertilization and reduced development time, as well as their ecological relevance, provide sea urchin embryos particularly useful for environmental risk assessment. In addition, is simple to obtain the gametes and promote *in vitro* fertilization, and the embryonic development and appearance of pluteus larvae, with four arms, occurs only 48 h at 20 ° C, without the need for feeding. In the Atlantic regions, the spawning of this species occurs in late spring, between the months of April and June. Nevertheless, with a marine algae based feeding and a temperature approximately of 18 °C, the adults from these regions can be kept fertile all year around.

Acclimatisation

2. Sea urchins can be collected in a clean rocky shore and transported to the laboratory in a portable icebox containing seawater or be purchased from recognised commercial sources, in which case they should be delivered to the laboratory within 24 h of dispatch.

In the laboratory the sea urchins can be kept in tanks with flowing seawater. The temperature of the water should be maintained around 18 °C to facilitate gonads maturation. The animals should be fed twice a week (at minimum) with various algae collected from natural sources. The feeding preferences of sea urchin are *Padina pavonica* and *Undaria pinnatifida*, among others. However, laboratory feeding of sea urchin is usually restricted to those species which are easily trapped in the sand, such as *Ulva* sp., *Sacchoriza polychides* and *Laminaria* sp.

Water

3. Control seawater is used for *in vitro* fertilization, preparation of test solutions and control incubations. Natural or synthetic salt water may be used. If natural seawater is used, it should be collected from an uncontaminated area and, prior to used, be passed through a filter effective to $\leq 0.2 \mu\text{m}$ to remove suspended particles, organisms and facultative pathogens from the water. If synthetic salt water is used, it should be prepared by dissolving the salts in deionized MilliQ water. All reagents must be of maximum quality (analytical grade or equivalent), and special care must be taken in the

trace metal impurity content of the salts. Commercial mixtures or “purified grade” salts are not suitable for larval rearing. The artificial seawater should be aged prior to use (24 to 48 h) and undergo continual aeration between preparation and use.

Test solutions

4. Test solutions of the chosen concentrations are prepared by dilution of a stock solution in seawater. If necessary, vehicles (e.g. organic solvents) of low toxicity may be used, in concentrations that should not exceed 0.1 mL/L. When vehicles are used, an additional control to that of seawater should be exposed to the same concentration of the vehicle as the used in the test solutions.

Test procedure

Conditions of exposure

1. Duration: 48 h;
2. Photoperiod: without light;
3. Temperature: 20 ± 0.2 °C;
4. Oxygen concentration: 2 mg/L;
5. Water parameters: pH 7.0 ($> 7.0 - < 8.5$), salinity 32 ± 1 ‰ ($> 31 - < 35$ ‰);
6. $\text{NH}_3/\text{L} < 40$ µg/L; $\text{H}_2\text{S} < 0.1$ mg/L;
7. The *P. lividus* embryos are very sensitive to changes in salinity (stenohaline) and pH. Also, unionized ammonia is highly toxic to sea urchin embryos while H_2S may also cause false positives, but unlike NH_3 , it is easily eliminated with gentle aeration. Water parameters should be measured at the beginning and end of the test period, on the test solutions specifically reserved for monitoring water quality. The concentration of test substance should be measured at a minimum at the beginning and at the end of the test, at the least in the control(s), and at the lowest and highest test concentration. Used microwell seawater can be collected and pooled to yield 1-mL for chemical analysis.

Test preparation

8. Females and males can usually be induced to spawn by one of two commonly used methods:
 - a. Osmotic-shock-induced spawning: 1 ml of 0.5M KCl is injected into the coelom with a small syringe by inserting the needle through the peristomal membrane surrounding the mouth on the oral side; spawning typically starts a few minutes after the injection. Females are inverted (oral side up) over a beaker containing seawater and left to release eggs. Sperm is aspirated “dry” from the gonopores of the males, since seawater activates the sperm cells. “Dry” spawning keeps the sperm in an inactive state, as they were in the gonad;
 - b. Gametes can also be obtained by excision of mature adults in an equatorial plane with a large knife. Care must be taken to employ safety gloves. A clean Pasteur pipette is inserted into the gonad and the eggs or sperm collected.

In these guidelines we will follow method b.

9. Prior to collecting the gametes, a small sample of sperm from each male should be transferred to an excavated slide together with a few drops of seawater and, after 15 minutes, observed under a microscope to assess the activity of the sperm. Male sea

urchins with the most motile sperm should be selected. The same procedure should be done with eggs to assess their quality. Batches of eggs with high proportions of abnormal eggs (e.g. those that fail to 'round up' after exposure to seawater) should be discarded.

10. After gametes analysis, eggs from a single female are collected and suspended in 90 mL of seawater into a 100 mL measuring cylinder until a dense orange suspension is formed. The beaker is then sealed with parafilm and gently inverted to homogenize the egg suspension.

11. A few microliters (2 to 3 drops) of undiluted sperm collected with a glass Pasteur pipette from a single male are added to the egg suspension, with gentle stirring provided by a plastic plunger. Too much sperm can cause polyspermy which should be avoided.

12. Four aliquots of 20 μ L of this mixed suspension are taken and examined microscopically using a mL counting chamber to assess egg population densities and fertilization success, which is indicated by the presence of a fertilization membrane (total number of eggs/mL + % of fertilized eggs/mL; respectively. $n \geq 100$). The percentage of fertilization should never be inferior to 90 %. Nevertheless, is recommended to performed toxicity tests when the fertilization rate is above 98 %.

Exposure

13. calculations are made to deliver 20 - 40 embryos/mL per well in 3 mL test solution (at least 5 concentrations in a geometric series with a factor preferably not exceeding 2.2), within 30 min after fertilization. 4 replicates per treatment (individual wells of a 3 mL microwell plate) should be performed. One blank (seawater) and a solvent control, if used, are run in addition to the test series with an equal number of embryos. The variation in the concentration of embryos in the various test solutions should be minimized by (i) keeping the embryo suspension well mixed by using a perforated plunger and (ii) carefully using a high-precision automatic pipet.

Note: If a useful prediction of the EC50 values is not available, it is usually desirable to conduct a range-finding test in which the test species is exposed to a control and three to five concentrations of the test chemical that differ by a factor of ten.

14. An additional series of control(s) replicates should be inoculated and then immediately fixed, for posterior counting to provide an estimate of the total numbers of larvae supplied to each test well (N) and to measure the eggs size at time zero (L_0).

15. The 24-well plates should be isolated with parafilm and incubated at 20 °C in the dark for 48 h.

16. At the end of the exposure time, before fixation, a control(s) well(s) should be assessed microscopically, using an appropriate counting chamber to determine the presence of well-developed larvae (pluteus larvae with 4 arms). It should also be verified that the maximum length of 35 individuals $> 308 \mu\text{m}$. This allows the early detection of manipulation errors or fixation date. If, after this time, this level of development and length has not been reached by the majority of the larvae, the bioassay must be repeated with biological material of highest quality.

17. The embryos are then fixed, in a fume cupboard, by adding two drops of 40 % formaldehyde and stored in appropriate closed vessels (e.g. 5 mL plastic vials).

Observations

18. Fixed individuals are observed with an inverted microscope using a graduated ocular or, preferably, using image analysis software (NIS or Q-Win type). In each well the maximum dimension of the first 35 individuals is taken (140 individuals per treatment) independently of their stage of development (fertilized eggs, embryos, prism larvae or pluteus larvae). For example, for the pluteus larvae, the maximum larval length is defined as the distance between the apex and the end of the post-oral arm.

19. The content from each replicate well should be placed in a counting chamber and all embryos and larvae counted. All larvae that have developed to a reasonably identifiable pluteus larva must be counted as normal, even if there are signs that the larva died after developing to the pluteus stage. Grossly deformed pluteus larvae or embryos that failed to develop beyond the prism stage must be counted as abnormal. Analyst training and experience are key factors in arriving at successful determinations of the difference between normal and abnormal pluteus larvae.

Note: because the volume of the counting cell is 1 mL, it might be necessary to prepare and count several slides to enumerate all embryos and larvae in each sample. Embryos and larvae usually sink after preservation, and it is frequently possible to discard most of the liquid before transferring the residual volume containing the organisms to the cell. Some larvae might not settle when preserved; it should therefore be determined periodically that embryos or larvae are not being discarded inadvertently.

Reference test

20. It may be useful to run a reference toxicant test to assess the biological quality of the test organisms. Intralaboratory control charts with reference toxicants (copper and zinc) indicated a coefficient of variation (*CV*) for percentage of normal larvae from 12 to 20 %.

Results

1. Size increase

For every incubation unit, experimental treatments, and controls, size increase is calculated as mean ($n = 35$) maximum dimension (L) minus mean egg size at time zero (L_0): $\Delta L' = L - L_0$.

Size increase values in the experimental treatments are expressed as percentages of the control. The PNR (percent net response) value is the response (length increase) in the test samples divided by the control:

$$PNR = \frac{\Delta L'}{\Delta L_c} \times 100$$

where:

ΔL_c is the average length increase of all control means.

For each treatment the average of the replicates and the standard error must be calculated.

2. Morphological abnormalities (MA)

For each test well in each treatment, including the control treatment(s), the percentage of the embryos that did not result in normal pluteus larvae should be calculated as follows:

$$\% \text{ abnormality} = 100(N - B)/N$$

where:

N = the average number of eggs in the control wells taken at time 0,

B = the number of normal pluteus larvae in the sample taken from that test well at the end of the test.

For each treatment the average of the replicates and the standard error must be calculated.

PNR_{MA} (the percentage of introduced embryos that did not result in normal pluteus larvae adjusted for the controls) should be calculated as follows:

$$\text{PNR}_{\text{MA}} = \frac{\% \text{ test abnormality} - \% \text{ control abnormality}}{100 - \% \text{ control abnormality}} \times 100$$

3. The data (total abnormally developed embryos and larvae, and size increase) should be used to determine:
 - d. The 48 h-EC50 values, the concentrations that results in an effect in 50 % of the exposed sea urchins after 48 h;
 - e. The 48 h-NOEC values, the highest no-observed effect concentration after 48 h;
 - f. The 48 h-LOEC values, the lowest observed effect concentration after 48 h.
4. The EC values should be determined using appropriate statistical procedures. Confidence limits (p=0.95) for the calculated EC value should be determined and quoted with the test results.
5. The NOEC and LOEC values should be determined using hypothesis testing. The data derived, as explained in this guidance, should be arc sine square root transformed prior to statistical treatment using analysis of variance.

Validity of the test

1. The percentage of fertilization should never be inferior to 90 %.
2. The length increase (ΔL_C) must be > 218 μm in fresh seawater and > 253 in artificial seawater.
3. At least 70 % of the embryos introduced into the control(s) treatment(s) must result in normal larvae at the end of the test.

References

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2.1.2. Test matrix: *Whole-sediment*

2.1.2.1. Amphipod sediment toxicity test

Based on ICES Techniques in Marine Environmental Sciences no. 28.

Principle of the test

This guideline describes a whole-sediment test using infaunal amphipods. The method description covers the use of *Corophium* spp., as this is the genus most commonly used in Europe. Adult *Corophium* are exposed to contaminated field sediments or chemically spiked sediments during short-term exposure. During this period, burrowing behaviour may be assessed by counting the number of amphipods on the sediment surface or actively swimming. At the end of the experiment, the amphipods are sieved from the sediment and the number of surviving animals is recorded. The objective is to determine the initial concentration which, in 10-day (d), kills 50 % of the exposed animals (LC50) and/or by the use of an appropriate analysis of variance technique to compare treatments with controls.

Test conditions

Equipment

- Sieve (approximately 500 µm);
- An orbital shaker or roller;
- Shaker bottles of the appropriate type and capacity (non-leaching plastic or glass, if organic contaminants are being tested, are generally the most practical);
- Test vessels; 1 L tall-form glass beakers are most commonly used, but any vessel of a suitable material is acceptable which permits a minimum sediment depth of 15 mm and a sediment overlying water volume ratio of approximately 0.2;
- Individual air stones;

- Automatic 5 ml dispensing pipette or 10 ml pipettes;
- Scoops for handling sediments;
- pH meter;
- Dissolved oxygen meter;
- Thermometer;
- Salinity or conductivity meter;
- Holding aquaria (approximate size of 10 L to 30 L);
- A balance that will weigh to two decimal places or to a precision greater than or equal to 1 % of the quantity being weighed;
- Clean 100 ml beakers (glass or of a suitable inert material). One beaker is required for each test vessel.

Species

1. Amphipods belonging to the genus *Corophium* are particularly useful for sediment risk assessment due to their burrowing activity. Either *Corophium volutator* or *Corophium arenarium* are acceptable, but the procedure can be used with any infaunal amphipod. The test animals must be collected from an area known to be relatively free from contamination. Adult *Corophium*, greater than 5 mm in length and smaller than 8 mm (excluding rostrum), should be used, to avoid problems of excessive background mortality. After sieving the animals should be transported to the laboratory in natural sea water.

Acclimatisation

2. In the laboratory, the animals are held in aquaria (about 10 to 30 L) in the presence of a small amount of detrital material under static or flow-through conditions, and should be used within 5-10 d. Mortalities during holding should be less than 10 %. Temperature, pH, salinity, and dissolved oxygen should all be recorded at regular intervals, and must stay within the range specified for the test. It is essential to measure the salinity at the point of collection. If the salinity is low, the animals should be acclimated to full salinity seawater (at least 25 ‰) at a maximum rate of approximately 3 per day.

Water

3. If natural salt water is used, it should be obtained from an uncontaminated area known to support a healthy, reproducing population of the test species or a comparable sensitive species. The fresh seawater should be prepared (passed through a filter effective to 0.45 µm or less to remove suspended particles, organisms and pathogens from the water) within 2 days of the test and stored in clean, covered containers at 4 ± 3 °C until sediment and water are added to the test vessels. Reconstituted salt water can be prepared by adding a commercially available sea salt or specified amounts of reagent- grade chemicals to high-quality water (e.g. deionized or distilled water). Reconstituted salt water should be intensively aerated before use, and aging for one to two weeks might be desirable. If a residue or precipitate is present, the solution should be filtered before use. Also, if necessary, the salinity should be reduced by diluting the sea water with high-quality deionized or distilled water, or raised by addition of clean filtered oceanic water or prepared brine.

Test solutions

4. In summary, chemicals for which a whole-sediment test is required are those which:
- are partly or completely classified as sinkers according to the “Standard European Behaviour Classification” (SEBC);
 - have a log P_{ow} of 4 or higher;
 - surface-active chemicals (unless full and reliable information indicates the opposite of assumed adsorbability);
 - are known to adsorb to particles (unless full and reliable information indicates the opposite of assumed adsorbability).

For more information on test solutions consult annex 2 (Preparation of test sediments).

Reference/base sediment

5. The reference sediment is used in the control treatment or as the base sediment for spiking purposes. It should have the following approximate characteristics:
- an organic content of between 0.5 and 4 %;
 - a silt/clay fraction (< 63 μm) of between 5 and 20 %;
 - a median grain size of 90 to 125 μm .

A muddy fine sand should be used, not a mud nor a coarse sand.

6. Sediment (approximately 40 kg wet weight or sufficient for the needs of the study) must be collected from an area known to be clean, and preferably from the same location from which the animals were collected. The aerobic layer of sediment (usually the top 5-10 cm) should be removed with a spade, and transferred to polythene bags or suitably cleaned vessels. The bags or containers should then be sealed, after excluding as much air as possible.

7. The sediment should be prepared and amended with the test substance as described in Annex 2.

Field contaminated sediment

8. All sediments used in tests must be collected and treated in a standardized manner. The provisions of ASTM E1391-90 (Standard guide for collection, storage, characterization and manipulation of sediments for toxicological testing), for instance, provide a suitable basis for a laboratory standard operating procedure.

9. The natural geochemical properties of test sediment collected from the field must be within the tolerance limits of the test species. Sediments should be collected by grab, taking samples from the upper 2-4 cm. These should be placed in polythene bags, excluding as much air as possible, and stored in the dark at 4 °C, no longer than two weeks before the initiation of the test, and must not be frozen or allowed to dry.

Test procedure

Conditions of exposure

- Duration: 10-d;
- Temperature: 15 ± 2 °C;
- Dissolved oxygen: ≥ 85 %;
- Water parameters: pH 7.5 - 8.5, full salinity seawater (at least 25 ‰);

5. The temperature, dissolved oxygen and pH should be measured at the start (before introduction of the test animals), after 24 hours (h), on one more occasion during, and at the end of the test. Salinity must be measured at the start and end of the test, (normally, a range of ± 4 during the experiment) and should be maintained close to that of day 0 value throughout the test. by the addition of distilled water. Salinity can readily be monitored by marking the final water level on the test beaker after the animals have been added. A 5 mm drop in level is equivalent to an approximately 2 increase in salinity. A change of this magnitude may be used as a practical threshold for restorative action, by addition of distilled water to the mark when required.

Test preparation

6. Approximately 150 mL of thoroughly homogenized field-collected sediment or spiked sediments, and control(s) sediment(s) (reference sediment and solvent if used) should be added to each test vessel. This should be sufficient to create a layer of 1.5-2 cm depth in a 1 L vessel. Care should be taken to ensure that the sediment suspension is evenly distributed between any replicates. The vessels should then be left overnight to allow the sediment to settle. Some surface water will appear during this procedure.

Note: The depth of sediment may vary in the test vessels. This is not critical except that the minimum depth must NOT be less than 15 mm, and the total sediment depth should not give rise to a sediment to water ratio of greater than approximately 0.2.

7. A disc of polythene cut to the internal diameter of the beaker should be lowered onto the sediment surface, by piercing the centre of the disc and threading a cable tie through the hole. Test sea water is then gently poured onto the polythene surface to bring the total

volume (i.e., sediment plus water) up to the 850 mL graduation mark. The polythene disc can then be gently removed.

8. After filling, each vessel is randomly allocated within the test area and the vessels left to allow any resuspended sediment to settle. The vessels are then covered, and aeration pipettes introduced through apertures in the covers. Gentle aeration is applied, and the vessels are observed to ensure that no sediment resuspension occurs. Test vessels must then be left undisturbed for at least 16 h before the addition of test animals.

Exposure

9. The minimum number of animals acceptable for the test is 20 per concentration and it is recommended that at least two replicates are used.

Note: If it is desired to compare control with treatment mortality then the degree of replication, should reflect this requirement (it is recommended that at least three replicates with a minimum of 20 animals per replicate be used).

10. Stock animals may be pipetted directly from the holding tank. Selection is made using an automatic or standard pipette, in which case the opening of the pipette should be 8 mm in diameter, the ends of which should be heat smoothed to avoid damaging the animals. Animals of a size of between approximately 5 and 8 mm are selected and randomly assigned to reference sea water in 100 ml beakers (one 100 ml beaker is required for each test vessel). When each beaker contains 10 (or 20, as appropriate)

Corophium, the volume of the beakers is adjusted to an appropriate mark (e.g. 40 ml), using reference sea water.

11. The aeration in the test vessels should be temporarily stopped and the test is initiated by randomly placing the groups of animals in the beakers into the test vessels. This is best achieved by gently moving the beaker to a horizontal position where the rim is under the water surface and then gently pouring out the contents of the beaker. Aeration should then be restored.

12. The test is continued for 10-d and termination is achieved by gently stirring up the sediment in the test beaker to form a slurry, and pouring the slurry into the 500 µm sieve, which is best achieved by keeping the sieve immersed in sea water. Any sediment left in the sieve should be gently washed away with reference sea water.

Observations

13. The number of animals alive and dead is recorded. Death is defined as the absence of movement after gentle stimulation with forceps. Dead animals may decompose or be consumed during the test and for this reason "missing" animals are presumed, and counted as, dead.

Note: records may be kept on the burrowing behaviour of the animals, although this may not be possible if the water in the test containers is turbid. Animals that are dead on the surface of the sediment are opaque in appearance. These mortalities should be recorded together with the number of animals that are not buried but may be seen swimming in the water column or browsing on the sediment surface. Where possible, dead animals should be removed.

Reference test

14. There is at present no established standard for reference toxicant effect. Fluoranthene was used as a standard hydrophobic reference chemical of sediment reworker bioassays. Limited experience with this chemical suggests that a 10-d LC50 of approximately 5-25 µg/Kg dry sediment is a reasonable initial compliance target.

Results

1. The endpoint of the test is mortality; which is calculated as the initial number of animals minus the number of surviving animals for each treatment at the end of the test.
2. The 10-d LC50 should be calculated using an appropriate statistical method. The raw mortality data must be provided in the final report.
3. For field-collected samples, the mortality values for each replicate should be arcsin-transformed, and the means of the transformed values compared by analysis of variance with the appropriate test for significance of difference to the control.

Validity of the test

Control mortality should not exceed 15 % during the test. Nevertheless, where higher mortalities are due to seasonal moribundity, it has been shown that a value of up to 20 % does not affect the relative response to different treatments. It is advised that Abbott's correction be applied prior to the LC50 calculation when mortality exceeds 15 %.

References

ASTM, 1991. Standard guide for conducting 10-day static sediment toxicity test with marine and estuarine amphipods, E1367-90. In: Annual Book of ASTM Standards, Water and Environmental Technology, Volume 11.04. American Society for Testing and Materials, Philadelphia, PA.

ASTM, 1991. Standard guide for collection, storage, characterization and manipulation of sediments for toxicological testing, E1391-90. In: Annual Book of ASTM Standards, Water and Environmental Technology, Volume 11.04. American Society for Testing and Materials, Philadelphia, PA.

OSPAR Commission, 2006. OSPAR Protocols on Methods for the Testing of Chemicals Used in the Offshore Oil Industry. ISBN 1-904426-99-9.

Roddie, B.D., Thain, J.E., 2001. Biological effects of contaminants: *Corophium* sp. sediment bioassay and toxicity test. ICES Techniques in Marine Environmental Sciences no. 28. 21 pp.

2.1.2.2. Polychaete sediment bioassay

Based on ICES Techniques in Marine Environmental Sciences no. 29.

Principle of the test

These guidelines describe procedures for obtaining laboratory data concerning the short-term adverse effects of contaminated sediment, or of a test substance experimentally added to uncontaminated sediment, on the polychaete *Arenicola marina* during static 10-day (d) exposures. Bioassay endpoints include both mortality and a non-lethal indication of effect (inhibition of casting). Healthy animals will bury themselves in the substrate almost immediately and cast regularly throughout the exposure period. Non-healthy animals will remain on the surface and die sometime during the exposure period, or they will bury themselves and cast little or not at all. In the latter case, dead worms may be found in the test sediments when they are sieved at the end of the experiment. The objectives are to determine the initial concentration which, in 10-d, kills 50 % of the exposed animals (LC50) or to determine statically significant mortality, and additionally, to determine the initial concentration which, in 10-d, inhibits casting and assign a relative casting ranking to each of the exposure concentrations or field-collected sediments.

Test conditions

Equipment

- Plastic tanks: sandwich-box type container, approximate size 200 mm (length) x 90 mm (width) x 90 mm (depth); sufficient to provide a substrate surface area of 150-200 cm² to a depth of 3 cm. If larger test containers are used, then the sediment volume must be adjusted accordingly, such that the sediment depth is always ≥ 3 cm;

- Approximately 2 mm sieve of plastic, nylon or stainless steel, preferably of a diameter of > 150 mm;
- Plastic dispensing pipette tip (ideally 200 µL tip);
- Shaker or roller for use only in spiking experiments (large orbital type that can be adapted to hold 2000 ml containers and to shake at 100 revolutions min⁻¹);
- Constant temperature facility;
- Eh probe and meter;
- Thermometer;
- Dissolved oxygen probe and meter;
- pH probe and meter;
- Salinity or conductivity meter;
- Top pan balance, 2 decimal places (for use only in spiking experiments).

Species

1. The polychaete *Arenicola marina* is a direct deposit feeder that is widely distributed in European coastal waters and on the east coast of North America. Animals may be collected from a clean intertidal shore or obtained from a bait supplier. An ideal size of worm is 1 g.

Acclimatisation

2. The preferred method of holding the animals is to keep them in a 20 L holding tank at a stocking density of approximately 200 animals with aerated flowing sea water, 5-10 ml min⁻¹, and a 3-cm layer of reference sediment on the bottom of the tank. However, animals can be kept for up to 7 days in static conditions.

Water

3. If natural salt water is used, it should be obtained from an uncontaminated area known to support a healthy, reproducing population of the test species or a comparable sensitive species. The fresh seawater should be prepared (passed through a filter effective to 0.45 µm or less to remove suspended particles, organisms and pathogens from the water) within 2 days of the test and stored in clean, covered containers at 4 ± 3 °C until sediment and water are added to the test vessels. Reconstituted salt water can be prepared by adding a commercially available sea salt or specified amounts of reagent- grade chemicals to high-quality water (e.g. deionized or distilled water). Reconstituted salt water should be intensively aerated before use, and aging for one to two weeks might be desirable. If a residue or precipitate is present, the solution should be filtered before use. Also, if necessary, the salinity should be reduced by diluting the sea water with high-quality deionized or distilled water, or raised by addition of clean filtered oceanic water or prepared brine.

Test solutions

4. In summary, chemicals for which a whole-sediment test is required are those which:

- a. are partly or completely classified as sinkers according to the “Standard European Behaviour Classification” (SEBC);
- b. have a log P_{ow} of 4 or higher;
- c. surface-active chemicals (unless full and reliable information indicates the opposite of assumed adsorbability);

- d. are known to adsorb to particles (unless full and reliable information indicates the opposite of assumed adsorbability).

For more information on test solutions consult annex 2 (Preparation of test sediments).

Reference/base sediment

5. The reference sediment is used in the control treatment or as the base sediment for spiking purposes. It should have the following approximate characteristics:

- a. an organic content of between 0.5 and 4 %;
- b. a silt/clay fraction (< 63 μm) of between 5 and 20 %;
- c. a median grain size of 90 to 125 μm .

6. For the test, a natural sediment should be collected, preferably a muddy sand from a foreshore known to be free of obvious contamination, down to a depth of 150 mm. The sediment should be transferred to polythene bags that should be sealed, after excluding as much air as possible.

7. Approximately 1 kg of sediment per test tank is required for each test vessel. Sediment preparation and amendment with the test substance should be performed as described in Annex 2.

Field contaminated sediment

8. All sediments used in tests must be collected and treated in a standardized manner. The provisions of ASTM E1391-90 (Standard guide for collection, storage, characterization and manipulation of sediments for toxicological testing), for instance, provide a suitable basis for a laboratory standard operating procedure.

9. These samples should be collected as appropriate, e.g., using a Day grab or box corer, but due care and consideration should be given to the amount of sample collected, the homogeneity, depth, degree of anoxia, and replication. This will depend on the objectives of the sampling, but the test requires a minimum of approximately 2 kg of wet sediment.

Test procedure

Conditions of exposure

1. Duration: 10-d;
2. Temperature: 15 ± 2 °C;
3. Dissolved oxygen: ≥ 85 %;
4. Water parameters: pH 7.5 - 8.5, full salinity seawater (30-35 ‰);
5. The temperature, dissolved oxygen and pH should be measured at the start (before introduction of the test animals), after 24 hours (h), on one more occasion during, and at the end of the test. Eh should be recorded immediately before the animals are placed in the tank and on the final day of the experiment. A reading at a depth of about 2 cm is taken at the front, middle, and rear of each tank. Salinity must be measured at the start and end of the test, (normally, a range of ± 4 during the experiment) and should be maintained close to that of day 0 value throughout the test. As constant aeration during the experiment causes some evaporation, dilution of sea water back to its initial salinity may be required. A 1 mm drop in level is equivalent to an increase of approximately 1 in salinity, and a change of this magnitude may be used as a practical threshold for

restorative action. This is best achieved by marking the seawater level on the tank on day 0, after addition of the animals, and, if necessary, daily topping up to this mark with distilled water.

Test preparation

6. Field-collected sediment or spiked sediments, and control(s) sediment(s) (reference sediment and solvent if used) should be tipped into a test tank and allowed to stand for 24 hours (h).

7. After that, clean sea water is added to each tank to a depth of about 3 cm above the sediment surface (this approximates to 500 ml per test tank). Aeration is then supplied to each tank, by fixing a small plastic dispensing pipette tip (ideally 200 µL tip) to the end of the aeration tubing, attaching it to the test vessel with a cable tie just above the sediment surface. Aeration should be adjusted to minimize disturbance to the sediment. The tanks should again be left to stand for 24 h.

Exposure

8. The animals are sieved from the holding tank and 5 individuals of a similar size are placed into each test container. At least two replicate tanks should be used for each test.

Observations

9. During the first 6 h, observations are made on the burying behaviour of the worms, although this may prove to be difficult as the animals may disturb the sediment whilst burying, giving poor visibility. After 24 h, the tanks are observed for mortalities at the sediment surface, which should be recorded, and the worms removed and replaced with new individuals. If any further animals fail to burrow, they should not be removed. A daily record should be kept of the numbers of dead or moribund animals on the substrate surface because this may be a contaminant related effect.

10. Healthy lugworms produce casts when active and feeding. To measure this activity, the number of casts in each tank should be noted and then all casts should be rubbed out (smoothed over gently with a spatula) and approximately 24 h later the casts should be counted again. This process should be repeated for the duration of the test.

11. After the animals have been exposed for 10-d, the sediment containing the worms in each tank is gently sieved in a bath of sea water and the number of animals surviving is recorded.

Note: Dead animals may decompose during the test and for this reason "missing" animals are presumed and counted as dead.

Reference test

12. The use of an internal reference chemical may be desirable and this will be determined by the nature of the investigation. Cadmium is frequently used for water-phase testing, but a "sediment-bound" toxicant may be preferable.

Results

1. The endpoint of the test is mortality; which is defined as the initial addition of animals minus the number of surviving animals for each treatment at the end of the test.

2. The 10-d LC50 should be calculated using an appropriate statistical method. The raw mortality data must be provided in the final report.

3. The mortality values for each replicate should be arcsin-transformed, and the means of the transformed values compared by analysis of variance with the appropriate test for significance of difference to the control. A hypothesis test may also be applied to the number of total casts for significance of difference in feeding behaviour to the control.

Validity of the test

The number of animals and replication used are small, therefore, control mortality should be 10 % or less.

References

ASTM, 1991. Standard guide for collection, storage, characterization and manipulation of sediments for toxicological testing, E1391-90. In: Annual Book of ASTM Standards, Water and Environmental Technology, Volume 11.04. American Society for Testing and Materials, Philadelphia, PA.

Thain, J.E., Bifield, S., 2001. Biological effects of contaminants: Sediment bioassay using the polychaete *Arenicola marina*. ICES Techniques in Marine Environmental Sciences no. 29. 16 pp.

Annexe 2. Preparation of test sediments

Sediment preparation

After sediment collection, on return to the laboratory the sediment should be sieved to 500 µm using reference seawater. Sieving in this way serves to adjust the interstitial salinity of the sediment and excludes any benthic organisms which might interfere with the test or eat the test animals. It is very important that the sediment is washed in a closed system and that the slurry is left to settle for 24 h before decanting the overlying water. The sediment should be carefully mixed before storage, into clean polythene bags. After as much air as possible has been excluded, the bags should be sealed and stored in the dark at 4 °C until they are required for use.

Chemical amendment of sediment

1. The quantity of sediment prepared per test substance concentration will depend on the size of the test vessels and the depth of sediment required (a minimum depth of 15 and 30 mm of sediment is required in each replicate for the 10-d test with *Corophium volutator* and *Arenicola marina*, respectively).

2. Immediately before the addition of a chemical or chemical solution, the base sediment must be thoroughly homogenised, and a sample of approximately 20 g wet weight removed and placed in an airtight container. This sample must be weighed (\pm 0.01 g) and then dried at approximately 60 °C for about 24 h. The dried sample must be cooled to room temperature in a dessicator and re-weighed. The ratio of the wet sample weight to dry sample weight will be calculated and entered on the study record.

Note: It is of primary importance that care be taken to avoid any alteration in the water content of the sediment between the time at which this determination is made and the time at which the test substance is added to the sediment.

3. The preparation of spiked sediments for whole-sediment toxicity tests is carried out in a manner dependent on the properties of the test substance:

- a. No floating or settled materials, liquid or solid (clear solution mixture, and homogeneous emulsion or fine/colloidal suspension) should be added to sediments as a solution or emulsion prepared in a small volume of seawater;
- b. Substances which are powders, are described as insoluble, or neutrally buoyant droplets (particles or floc); floating, but no settled, liquids or solids; settled, but no floating, liquids or solids; and floating and settled liquids or solids should be added initially to a small quantity of dried sediment and mixed thoroughly before mixing with a larger volume of wet sediment;
- c. Insoluble or poorly-soluble substances should be dissolved in a suitable organic solvent such as methanol or acetone before addition to dried sediment. Where a solvent is used, additional control sediments must be prepared at least at the highest concentration of solvent used in the substance treatments.

4. Test chemical concentrations may be prepared either:

- a. as nominal concentrations per unit wet weight of the base sediment, and later corrected using the measured wet weight/dry weight ratio to units of mg/kg dry weight;
- b. as nominal concentrations per unit dry weight, by calculating the appropriate addition rate per unit wet weight on the basis of the measured wet weight/dry weight ratio.

Note: calculation of the required quantity of test substance must take into account the weight of any dry sediment used in preliminary preparation. Where the test substance is prepared as an aqueous suspension or emulsion, the volume of water used should be kept to a minimum.

5. If the test is intended to allow calculation of the LC50, the animals should be exposed to five (or more concentrations) of the test chemical in a geometric series, in which the test concentrations should bracket the predicted LC50, based on the results of a test on the same or a similar test chemical with the same or a similar species. If a useful prediction is not available, it is usually desirable to conduct a range-finding test in which the test species is exposed to a control and three or more concentrations of the test material that differ by a factor of ten.

Mixing of test substance with sediment

1. The test substance and carrier medium should be added to the appropriate weight of wet sediment in a suitable container (e.g. a polythene or polypropylene bottle) of a suitable volume (e.g. 1 or 2 L for a *Corophium* test and 2 L for a *Arenicola* test as referred to above); in general, a vessel volume of approximately twice the volume of sediment is acceptable. A clean spatula (e.g. stainless steel or polythene) should be used to initially disperse the test substance and carrier through the sediment.

Note: each exposure concentration should be prepared separately and no attempt should be made to serially dilute any sediment once contaminated with a test compound.

2. Sufficient clean seawater is then added to create a freely-flowing slurry. Care must be taken to minimise the volume of water added, but the quantity must be sufficient to allow the mixture to flow freely when the container is inverted or shaken.
3. The container will be labelled with the study number, the test substance number and the nominal concentration, and placed horizontally on an orbital shaker (with a displacement of at least 30 mm) at about 150 rpm for approximately 3 h. The purpose of this procedure is to ensure that the test substance is evenly distributed throughout the sediment matrix. A roller may also be used for mixing.
4. Each container should be removed from the shaking apparatus and finally shaken thoroughly by hand, with at least five inversions, and the test operator should establish that the preparation moves freely and that no unmixed residues remain adhered to the walls of the container. This procedure must be carried out for each container immediately before the contents are dispensed to the appropriate replicate vessels.
5. The test medium should be dispensed in small aliquots to each replicate test vessel in turn, to minimize bias in the chemical content or particle-size distribution of the medium between vessels. Any supernatant water present must be included in the test system, and must be equally distributed between vessels before the solid material is dispensed.
6. If difficulty is encountered in dispensing the solid phase, a clean spatula may be used to manipulate the material; in this case, it is essential that a new spatula be used for each test substance, and that each test substance be dispensed in order from lowest to highest nominal concentration.
7. Sediment preparation containers must be used only once and should be disposed of as soon as practicable after dispensing the solid phase to the test vessels.
8. Preparation of the test system will then proceed in accordance with the guidelines.

2.2. Biomarkers

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¹Authors contribute equitably to the sub-chapter.

Biomarkers can be considered “functional measures of exposure to stressors expressed at the sub-organismal, physiological or behavioural level” (Galloway, 2006).

A wide range of biomarkers can be used in environmental monitoring as sensitive early warning indicators of contamination and ecosystem health. So, their use is essential in order to anticipate potential impacts at higher levels of biological organization (e.g. population, community, and ecosystem).

Therefore, the biochemical responses of organisms (at sub-individual and individual level) should be used as powerful tools for monitoring the biological effects of Hazardous and Noxious Substance (HNS) in a spill event. Furthermore, the battery of biomarkers that should be applied in the event of a HNS spill (for 24 HNS considered

priority) can be found in Table 3. These parameters are well established as relevant indices to draw a diagnostic on the ecological status of the environment.

Table 3 – Recommended battery of biomarkers to apply in the event of a HNS spill (for 24 HNS considered priority) (X – means that the protocol should be applied).

Priority HNS	Biomarkers – Protocols*							
	SOD	CAT	LPO	AChE	vtg	Liver histopathology	MN	LMS - NRR
Benzene ¹	X	X	X			X	X	X
Cyclohexane ¹	X	X	X			X		X
Heptane ¹	X	X	X			X		X
Hexane ¹	X	X	X	X		X		X
Toluene ¹	X	X	X	X		X		X
1-Nonene ²	X	X	X			X		X
Octane ²	X	X	X			X		X
Styrene ²	X	X	X			X	X	X
m-Xylene ²	X	X	X			X		X
Butyl acrylate ³	X	X	X			X		X
Aniline ⁴	X	X	X			X	X	X
Cyclohexylbenzene ⁵	X	X	X			X		X
Pentylbenzene ⁵	X	X	X			X		X
Decanoic acid ⁶	X	X	X			X		X
Di (2-ethylhexyl) adipate ⁶	X	X	X			X		X
1-Dodecanol ⁶	X	X	X			X		X
Isononanol ⁶	X	X	X			X		X
1-Nonanol ⁶	X	X	X			X		X
4-Nonylphenol ⁶	X	X	X		X	X		X
Tetrachloroethylene ⁷	X	X	X			X	X	X
m-Cresol ⁸	X	X	X			X		X
Nitrobenzene ⁸	X	X	X			X	X	X
Trichloroethene ⁸	X	X	X			X	X	X
Acrylonitrile ⁹	X	X	X			X	X	X

* **SOD** - Superoxide Dismutase; **CAT** - Catalase; **LPO** - Lipid Peroxidation; **AChE** – Acetylcholinesterase; vtg - Vitellogenin; **MN** - Micronuclei; **LMS - NRR** - Lysosomal membrane stability - Neutro Red Retention assay.

Behaviour in seawater: 1 - Evaporator; 2 - Floater/evaporator; 3 - Floater/evaporator/dissolver; 4 - Floater/dissolver; 5 - Floater; 6 - Floater persistent; 7 - Sinker; 8 - Sinker/dissolver; 9 - Dissolver/evaporator.

The following protocols are focused on invertebrate and vertebrate species such as mussels and fishes.

References

Galloway, T.S., 2006. Biomarkers in environmental and human health risk assessment. *Mar Pollut Bull.* 53: 606–613.

2.2.1. Determination of the enzymatic activity of Superoxide dismutase (SOD) in the liver of invertebrate and vertebrate species

Principle of the test

Superoxide dismutase (SOD) is an antioxidant enzyme that can be found in the mitochondria and cytoplasm of the cells. Analysis of SOD hepatic activity can be used as a biomarker of exposure to pollutants causing oxidative stress (e.g. metals). Therefore, the following protocol allows to assess the presence of oxidative stress in invertebrate and vertebrate species (e.g. fish, mussels) by the determination of the SOD hepatic activity. SOD activity should be determined by an indirect method involving the inhibition of cytochrome *c* reduction in the mitochondrial fraction. In this method SOD competes with cytochrome *c* for the superoxide anion generated by the xanthine and xanthine oxidase reaction.

Material

Equipment

- Graduated cylinders;
- Glass beakers;
- Precision balance;
- Homogenizer;
- Centrifuge;
- Microtubes (1.5 ml, 2.0 ml);
- Micropipettes (e.g. 10-100 μ l, 100-1000 μ l);
- Multi-channel pipettes (e.g. 10-50 μ l, 30-300 μ l);
- Transparent 96-well microplates;
- Microplate reader with 550 nm filter.

Reagents

All reagents must be brought to room or controlled temperature prior to use.

- 100 mM Potassium phosphate buffer (pH 7.4);
- 150 mM Potassium chloride (KCl);
- 1 mM Dithiothreitol (DTT);
- 0.1 mM Phenylmethylsulfonyl fluoride (PMSF);
- 1 mM Disodic ethylenediaminetetra acetic acid (Na₂EDTA);
- Bovine serum albumin (BSA);
- 50 mM Sodium phosphate buffer (pH=7.8);
- 0.1 mM Na₂EDTA;
- 50 μ M Xanthine;
- 5.2 mU/ml Xanthine oxidase;
- 18 μ M Cytochrome *c*;

- SOD standards.

Test procedure

1. The animals should be captured and killed to remove the liver. Fish should be anesthetized in tricaine methanesulfonate (MS 222) at a concentration of 300-400 mg/l, molluscs in 7 % magnesium chloride.
2. If necessary, the liver should be immediately frozen in liquid nitrogen and subsequently stored at -80 °C until further analysis.
3. Liver should be homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4), 150 mM KCl, 1 mM DTT, 0.1 mM PMSF and 1 mM Na₂EDTA.
4. Mitochondrial fractions should be obtained after centrifugation at 15000 rcf for 20 min at 4 °C.
5. SOD activity should be determined in the mitochondrial fraction by the degree of inhibition of cytochrome *c* reduction via the measurement of the absorbance at 550 nm.
 - 5.1 In the assay, the concentration of the reactives should be as follows: sodium phosphate buffer 50 mM, pH=7.8, with Na₂EDTA 0.1 mM, xanthine 50 μM, xanthine oxidase 5.2 mU/ml and cytochrome *c* 18 μM.
 - 5.2 Two hundred and fifty microliters of this mixture should react with the sample (protein concentration should be 4 mg/ml*) diluted in phosphate buffer to a final volume of 300 μl.
 - 5.3 To obtain linearity, the volumes of sample and phosphate buffer per well should be adjusted for each sample.
 - 5.4 The absorbance values should be recorded every 20 seconds for 2 minutes.
6. SOD standards should be used in each assay to calculate the activity in SOD units.
7. SOD activity should be performed in triplicate for each sample.

* Protein content should be measured by the Lowry method using bovine serum albumin (BSA) as a standard.

Results

The greater the reduction of cytochrome *c*, the smaller the amount of SODs present (i.e., high absorbance values indicate low SOD values).

The activity should be given in SOD units (1 SOD unit = 50 % inhibition of the reduction of cytochrome *c*) per milligramme of protein (U/mg protein).

References

Capela, R., Raimundo, J., Santos, M.M., Caetano, M., Micaelo, C., Vale, C., Guimarães, L., Reis-Henriques, M.A., 2016. The use of biomarkers as integrative tools for transitional water bodies monitoring in the Water Framework Directive context - A holistic approach in Minho river transitional waters. *Sci Total Environ.* 539: 85-96.

Cunha, V., Santos, M.M., Moradas-Ferreira, P., Ferreira, M., 2016. Simvastatin effects on detoxification mechanisms in *Danio rerio* embryos. *Environ Sci Pollut Res Int.* 23: 10615-10629.

Ferreira, M., Caetano, M., Antunes, P., Costa, J., Gil, O., Bandarra, N., Pousão-Ferreira, P., Vale, C., Reis-Henriques, M.A., 2010. Assessment of contaminants and biomarkers of exposure in wild and farmed seabass. *Ecotoxicol Environ Saf* 73: 579-588.

Ferreira, M., Caetano, M., Costa, J., Pousão-Ferreira, P., Vale, C., Reis-Henriques, M.A., 2008. Metal accumulation and oxidative stress responses in, cultured and wild, white seabream from Northwest Atlantic. *Sci Total Environ.* 407: 638-646.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J Biol Chem.* 193: 265-275.

2.2.2. Determination of the enzymatic activity of Catalase (CAT) in the liver of invertebrate and vertebrate species

Principle of the test

Catalase (CAT) is an antioxidant enzyme that can be found in the peroxisomes of the cells. Analysis of CAT hepatic activity can be used as a biomarker of exposure to pollutants causing oxidative stress (e.g. metals). Therefore, the following protocol allows to measure the presence of oxidative stress in invertebrate and vertebrate species (e.g. fish, mussels) by the determination of the CAT hepatic activity. CAT activity should be determined by measuring the consumption of H₂O₂ at 240 nm.

Material

Equipment

- Graduated cylinders;
- Glass beakers;
- Precision balance;
- Homogenizer;
- Centrifuge;
- Microtubes (1.5 ml, 2.0 ml);
- Micropipettes (e.g. 10-100 µl, 100-1000 µl);
- Cuvettes of 1 ml;
- Spectrophotometer.

Reagents

All reagents must be brought to room or controlled temperature prior to use.

- 100 mM Potassium phosphate buffer (pH 7.4);
- 150 mM Potassium chloride (KCl);

- 1 mM Dithiothreitol (DTT);
- 0.1 mM Phenylmethylsulfonyl fluoride (PMSF);
- 1 mM Disodic ethylenediaminetetra acetic acid (Na₂EDTA);
- Bovine serum albumin (BSA);
- 65 mM Potassium phosphate buffer (pH=7.8);
- 15.5 mM H₂O₂;
- 0.01 % Triton X-100.

Test procedure

1. The animals should be captured and killed to remove the liver. Fish should be anesthetized in tricaine methanesulfonate (MS 222) at a concentration exceeding 300-400 mg/l, molluscs in 7 % magnesium chloride.
2. If necessary, the liver should be immediately frozen in liquid nitrogen and subsequently stored at -80 °C until further analysis.
3. Liver should be homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4), 150 mM KCl, 1 mM DTT, 0.1 mM PMSF and 1 mM Na₂EDTA.
4. Mitochondrial fractions should be obtained after centrifugation at 15000 rcf for 20 min at 4 °C.
5. CAT activity should be determined by measuring the consumption of H₂O₂ at 240 nm (extinction coefficient 40 L mol⁻¹ cm⁻¹) in a reaction mixture containing 65 mM potassium phosphate buffer, pH=7.8, 15.5 mM H₂O₂ and 0.01 % Triton X-100.
 - 5.1 The reaction should be started by the addition of the sample. Thus, in the cuvette, 950 µl of reaction mixture should be added to the sample (protein concentration should be 4 mg/ml*) and diluted in phosphate buffer to a final volume of 1000 µl.
 - 5.2 To obtain linearity, the volumes of sample and phosphate buffer should be adjusted for each sample.
 - 5.3 The absorbance values should be recorded every 10 seconds for 2 minutes. Through these values, the slope (that reflects the activity of the enzyme) should be calculated. Note: The higher the slope, the greater the activity.
6. CAT activity should be performed in triplicate for each sample.

* Protein content should be measured through the Lowry method using bovine serum albumin (BSA) as a standard.

Results

CAT activity should be calculated using the following formula:

$$CAT\ activity\ (\mu mol\ min^{-1}\ mg\ protein^{-1}) = \frac{Slope \times Vol_T}{\epsilon \times [protein] \times Vol_s} \times 1000$$

where:

Slope (min⁻¹)

Vol_T = total volume (1 ml);
 ϵ = extinction coefficient = $40 \text{ L mol}^{-1} \text{ cm}^{-1}$;
[protein] = concentration of protein = 4 mg ml^{-1} ;
 Vol_s = sample volume (ml).

References

- Capela, R., Raimundo, J., Santos, M.M., Caetano, M., Micaelo, C., Vale, C., Guimarães, L., Reis-Henriques, M.A., 2016. The use of biomarkers as integrative tools for transitional water bodies monitoring in the Water Framework Directive context - A holistic approach in Minho river transitional waters. *Sci Total Environ.* 539: 85-96.
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- Ferreira, M., Caetano, M., Costa, J., Pousão-Ferreira, P., Vale, C., Reis-Henriques, M.A., 2008. Metal accumulation and oxidative stress responses in, cultured and wild, white seabream from Northwest Atlantic. *Sci Total Environ.* 407: 638-646.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J Biol Chem.* 193: 265-275.

2.2.3. Determination of Lipid Peroxidation (LPO) in the liver of invertebrate and vertebrate species

Principle of the test

The measurement of lipid peroxidation (LPO) can be used as an effect biomarker indicative of oxidative stress damage as a consequence of a contaminant exposure (e.g. metal exposure). The LPO occurs with free radical generation and results in the production of malondialdehyde (MDA). Therefore, the following protocol allows to determine the LPO, measured as MDA levels, in the liver of different invertebrate and vertebrate species (e.g. fish, mussels). Such parameter should be determined by the thiobarbituric acid (TBA) method.

Material

Equipment

- Graduated cylinders;
- Glass beakers;
- Precision balance;

- Homogenizer;
- Centrifuge;
- Microtubes (1.5 ml, 2.0 ml);
- Micropipettes (e.g. 10-100 μ l, 100-1000 μ l);
- Cuvettes;
- Spectrophotometer.

Reagents

All reagents must be brought to room or controlled temperature prior to use.

- 100 mM Potassium phosphate buffer (pH 7.4);
- 150 mM Potassium chloride (KCl);
- 1 mM Dithiothreitol (DTT);
- 0.1 mM Phenylmethylsulfonyl fluoride (PMSF);
- 1 mM Disodic ethylenediaminetetra acetic acid (Na_2EDTA);
- Bovine serum albumin (BSA);
- 100 % Trichloroacetic acid (TCA);
- 1 % Thiobarbituric acid (TBA);
- 0.05 M NaOH;
- 0.025 % Butylated hydroxytoluene (BHT).

Test procedure

1. The animals should be captured and killed to remove the liver. Fish should be anesthetized in tricaine methanesulfonate (MS 222) at a concentration exceeding 300-400 mg/l, molluscs in 7 % magnesium chloride.
2. If necessary, the liver should be immediately frozen in liquid nitrogen and subsequently stored at $-80\text{ }^\circ\text{C}$ until further analysis.
3. Liver should be homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4), 150 mM KCl, 1 mM DTT, 0.1 mM PMSF and 1 mM Na_2EDTA .
4. Mitochondrial fractions should be obtained after centrifugation at $15000 \times g$ for 20 min at $4\text{ }^\circ\text{C}$.
5. Protein content in the fractions should be assayed by the Lowry method using bovine serum albumin (BSA) as a standard.
6. The peroxidative damage to lipids should be determined by the TBA method.
 - 6.1 The liver homogenate should be incubated with TCA 100 %;
 - 6.2 After new centrifugation (at $5000 \times g$ for 20 min at $4\text{ }^\circ\text{C}$), the supernatant should be incubated at $100\text{ }^\circ\text{C}$, for 30 min, with TBA 1 %, NaOH 0.05 M and BHT 0.025 %.
 - 6.3 The absorbance should be measured at 532 nm.
7. LOP should be performed in triplicate for each sample.

Results

LPO should be expressed as nanomoles of MDA equivalents per mg of protein (nmol MDA/mg protein).

References

- Capela, R., Raimundo, J., Santos, M.M., Caetano, M., Micaelo, C., Vale, C., Guimarães, L., Reis-Henriques, M.A., 2016. The use of biomarkers as integrative tools for transitional water bodies monitoring in the Water Framework Directive context - A holistic approach in Minho river transitional waters. *Sci Total Environ.* 539: 85-96.
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- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. *J Biol Chem.* 193: 265-275.

2.2.4. Assessment of the enzymatic activity of acetylcholinesterase in invertebrate and vertebrate tissues

Principle of the test

Acetylcholinesterase (AChE) is present in most animals and is responsible for the quick hydrolytic degradation of the neurotransmitter acetylcholine (ACh) into the inactive products choline and acetic acid. The role of AChE in cholinergic transmission is to regulate the nervous transmission by reducing the concentration of ACh in the junction through AChE-catalysed hydrolysis of ACh. When AChE is inactivated by a toxic compound, the enzyme is no longer able to hydrolyse ACh and consequently, the concentration of ACh remains high in the junction. Continuous stimulation of the muscle or nerve fibre then occurs, resulting in tetany and eventually paralysis and death. Therefore, AChE can be used as a biomarker of the effects of neurotoxic compounds with ability to inactivate this system (e.g. organophosphates, carbamates, etc).

The following protocol allows to determine the enzymatic activity of AChE in different invertebrate and vertebrate tissues (e.g. fish muscle and brain, abdominal muscle of crustaceans, adductor muscle and gills of bivalve molluscs). This determination should be based on Ellman's method (Ellman et al., 1961) adapted to a microplate reader.

Material

Equipment

- Precision balance;
- Beakers (10, 250 and 500 mL);
- Ultra Turrax homogeneizer;
- Centrifuge;
- Microtubes (1.5 ml and 2.0 ml);
- Pipettes (10-100 μ l, 100-1000 μ l and 1-10 ml);
- Multi-channel pipettes (10-50 μ l and 50-300 μ l);
- Transparent 96-well microplates;
- Microplate reader with 412 nm filter.

Reagents

All reagents must be brought to room or controlled temperature prior to use.

- 0.02 M Phosphate buffer (pH=7.0);
- 0.1 % Triton X 100;
- Bovine serum albumin (BSA);
- Bradford's reagent (phosphoric acid, methanol, and Coomassie brilliant blue);
Note: Dilutions of the concentrated Bradford's reagent (commercially available) with distilled water must be utilized within two weeks. The colour development is stable for one hour.
- 0.1 M Acetylthiocholine iodide (ACTC) (2.6 mM final concentration) -
Dessicated ACTC must be stored at temperatures lower than 0 °C and the 0.1 M ACTC stock solution must be prepared in distilled water immediately prior to use.
- 0.01 M Dithiobisnitrobenzoate (DTNB) (0.5 mM final concentration) that should be prepared in 0.1 M TRIS, pH=8 buffer and can be used for several days when stored at 4 °C.

Test procedure

1. An extraction should be performed on fresh or frozen tissue (0.1 to 1 g)⁶ using 0.02 M phosphate buffer (pH=7.0) containing 0.1 % Triton X 100;
 - 1.1 The tissue should be homogenized 1/1 to 1/4 (w/v) depending on the species or organ (e.g. fish brain tissue has a higher specific AChE activity than muscle) for one minute using an Ultra Turrax.
2. Extracts should be then centrifuged at 10,000 x g for 20 minutes and an aliquot of the supernatant should be used in the assay. These supernatants can be stored at -20 °C or below without significant loss of activity.
3. Then, protein should be determined:

⁶ Assays must be performed on individuals of known age (size) and sex. The recommended number of samples required is a minimum of six to ten animals per site.

- 3.1 The method described by Bradford (1976) can be used for quantitative determination using bovine serum albumin (BSA) as the protein standard. This method should be adapted to be used with a microplate reader.
 - 3.2 For each microplate well, 280 μl of Bradford's reagent should be added to 100 μl of an appropriate dilution of the sample.
 - 3.3 A protein standard curve (1 to 10 $\mu\text{g } \mu\text{l}^{-1}$) should be prepared each time the assay is performed by diluting lyophilized bovine serum albumin with distilled water.
 - 3.4 Rehydrated protein can be stored up to 60 days at 4 $^{\circ}\text{C}$.
 - 3.5 Absorbance should be read at 595 nm and the sample concentration should be calculated from the standard curve.
4. AChE activity should be determined by the Ellman's method⁷ (Ellman et al., 1961) adapted for use with a microplate reader.
- 4.1 To each well of the microplate, 340 μl of 0.02 M phosphate buffer (pH=7), 20 μl of 0.01 M DTNB (0.5 mM final concentration) and 10 μl of supernatant should be added in succession.
 - 4.2 After five minutes' incubation to allow the DTNB to react with the sulfhydryl groups of the amino acids in the sample, 10 μl of 0.1 M ACTC (2.6 mM final concentration) should be added to begin the enzymatic reaction.
(Note: Make sure that the final concentration of 2.6 mM is maintained accurately because AChE is subject to substrate inhibition at 10 mM final concentration of ACTC.)
 - 4.3 The enzyme kinetics should be monitored on a microplate reader at 412 nm (from 405-420 nm).
 - 4.4 Wells without enzymatic extract (buffer + DTNB + ACTC) should be used to estimate the spontaneous hydrolysis of ACTC.
 - 4.5 All assays can be easily duplicated using a microplate.

Results

Calculation of AChE activity

- The change in absorbance (OD) per minute should be determined by subtracting the absorbance due to non-enzymatic hydrolysis of substrate from the absorbance increase per minute measured for the sample.
- Enzyme activity should be then expressed in Units of activity, where one Unit should be defined as the amount of enzyme which catalyses the hydrolysis of 1 μmole of acetylthiocholine per minute per mg protein, and should be calculated using the following formula:

$$AChE \text{ activity } (\mu\text{mol ACTC min}^{-1} \text{ mg protein}^{-1}) = \frac{\Delta A_{412} \times Vol_T \times 1000}{1.36 \times 10^4 \times \text{lightpath} \times Vol_s \times [\text{protein}]}$$

where:

⁷ It uses acetylthiocholine iodide (ACTC) as a specific substrate. Ellman's method is based on the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate (DTNB) ion.

ΔA_{412} = change in absorbance (OD) per min, corrected for spontaneous hydrolysis;
 Vol_T = total assay volume (0.380 ml);
 1.36×10^4 = extinction coefficient of TNB ($M^{-1} \text{ cm}^{-1}$);
Light path = microplate well depth (1 cm);
 Vol_s = sample volume (in ml);
[protein] = concentration of protein in the enzymatic extract (mg ml^{-1}).

References

- Bocquené, G., Galgani, F., 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences no. 22. 12 pp.
- Bradford, M., 1976. A rapid and sensitive assay of protein utilizing the principle of dye binding. *Anal Biochem.* 77: 248-264.
- Ellman, G.L., Courtney, K.O., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* 7: 88-95.
- Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soares, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. *Chemosphere* 32:727-738.

2.2.5. Analysis of vitellogenin (*vtg*) gene expression in fishes

Principle of the test

There is a growing concern about the presence of estrogenic compounds (both steroids and xenoestrogens) in the aquatic environment because of the clear indications of endocrine disruption in wild fish populations. Vitellogenin (*vtg*) which encodes a protein that acts as a precursor of egg yolk, is well characterised as estrogen-inducible gene. Thus, vitellogenin (*vtg*) should be included as a gene of interest in studies on the effects of environmental estrogens. Furthermore, the analysis of vitellogenin (*vtg*) gene expression in fishes can be used as a sensitive and reliable indicator of estrogenic chemicals exposure (or a biomarker of endocrine disruption).

The following protocol allows to measure the hepatic gene expression of vitellogenin (*vtg*) in fishes using real-time reverse transcription polymerase chain reaction (qPCR).

Material

Equipment

- Beads;
- Threaded tubes (2.0 ml);
- Microtubes (0.2 ml and 1.5 ml);
- Micropipettes (e.g. 1-10 μl , 10-100 μl);

- IQTM5 PCR Detection System of BioRad (or other);
- Clear 96-well plates.

Reagents

- RNAlater;
- Illustra RNAspin Mini RNA Isolation Kit of GE Healthcare;
- RiboGreen RNA Quantitation Reagent Kit of Invitrogen;
- Kit Iscript cDNA Synthesis (Biorad);
- Vector pGEM-T and pGEM-T easy vector systems (Promega);
- NovaBlue competent cells (Novagen);
- iQTM SYBR[®] Green Supermix (BioRad).

Test procedure

1. Fish should be captured and killed to remove the liver.
2. If necessary, the liver should be immediately frozen in liquid nitrogen and subsequently stored at -80 °C until RNA extraction for subsequent *vtg* gene expression analysis. Alternatively, the liver can be stored in RNA lather (10 times weigh/volume). As little as 50 mg is enough to perform the assay.
3. RNA extraction of the liver should be performed through the illustra RNAspin Mini RNA Isolation kit of GE Healthcare, using beads (2 beads for sample, 5800 ×g 2x 15 seconds) for the homogenisation of the tissue, following manufacturer's instructions.
4. RNA concentration should be determined with RiboGreen RNA Quantitation Reagent Kit of Invitrogen.
5. 1 µg of RNA should be used for the synthesis of cDNA using the kit Iscript cDNA Synthesis (Biorad), according to the manufacturer's specifications.
6. Primers for each *vtg* gene were designed using Primer 3 *software* (available at: <http://www.ncbi.nlm.nih.gov/> or equivalent software) based on the sequences already identified.

6.1 Primers sequence designed using Primer 3 *software* for the plaice:

PlaiceVTG_RT_FOW: 5'- TCCAAGAAAACCCAATCCTG - 3'

PlaiceVTG_RT_REW: 5'- AGCCTGGACAGCAAGTTCAT - 3'

Notes (Ferreira et al., 2013):

a) The primers were designed using an online NCBI-BLAST database (NCBI-nucleotideBLAST; available at: <http://www.ncbi.nlm.nih.gov/BLAST/>) for VTG I isolation in the sentinel fish species *Lipophrys pholis* (shanny, an intertidal *Blenniidae*) as follows:

VTGF1: 5'- CAGGTNTTRGCWCARGAYTG - 3'

VTGR2 5'- AGRMASMACCCAGGARTGVGC - 3'.

b) The primers sequence was designed using an online NCBI-BLAST database (NCBI-nucleotideBLAST; available at: <http://www.ncbi.nlm.nih.gov/BLAST/>) for β-actin (housekeeping gene) in *Spaurus aurata* as follows:

Forward 5'- GATCATGTTTCGAGACCTTCAA - 3'
Reverse 5'- TCCAATCCAGACAGAGTATTTACG - 3'R.

7. The product of each PCR should be confirmed by sequencing.
 - 7.1 The PCR product with the expected band size should be purified and cloned using the vector pGEM-T and pGEM-T easy vector systems (Promega);
 - 7.2 Then, the NovaBlue competent cells (Novagen) should be transformed;
 - 7.3 Finally, after culture in liquid medium, the PCR product with the expected band size should be isolated and sequenced, for example, on StabVida (Portugal).
8. The real-time quantitative PCR (qPCR) should be performed with the IQTM5 PCR Detection System of BioRad (or equivalent).
9. An efficiency curve should be made for the *vlg* gene to be tested and the optimal concentration of the primers pair used should be tested based on the lower CT's at higher levels of fluorescence.
 - 9.1 The efficiency curve should be performed through 6 successive dilutions of the template (5x dilution), with an initial concentration of 50 ng/μl.
 - 9.2 The slope and the regression curve must be calculated.
10. An amplification volume of 20 μl containing 10 μl of iQTM SYBR[®] Green Supermix (BioRad), 0.6 μl of each primer (concentration previously tested), 4 μl of cDNA and 4.8 μl of autoclaved MiliQ water should be used. All reactions must be performed with duplicates.
- 11 - The conditions of qRT-PCR should be as follows: 1 cycle at 95 °C for 20 min, 40 cycles at 95 °C (10 seconds), melting temperature 58 °C (30 seconds) and 72 °C (30 seconds). The melting curve should be performed to verify the formation of secondary products (from 57 °C to 95 °C, 15 seconds).
- 12 - Expression quantification should be done through normalisation with a reference gene (b-actin) and through the applicable mathematical model.

Results

Results can be expressed as fold-increase in *vlg* expression from the control.

References

- Ferreira, F., Monteiro, N.M., Vieira, M.N., Reis-Henriques, M.A., Castro, L.F.C., Santos, M.M., 2013. A real-time PCR assay for differential expression of vitellogenin I and II genes in the liver of the sentinel fish species *Lipophrys pholis*. *Toxicol Mech Methods*. 23: 591-597.
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- Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 29: e45.

2.2.6. Analysis of liver histopathology in fishes

Principle of the test

Toxicopathic liver lesions in fish can be used as histopathological biomarkers of contaminant effects and exposure (e.g. carcinogen exposure) in environmental monitoring programmes. Thus, these lesions can be useful as sensitive and reliable indicators for the health assessment of wild fish populations.

The following protocol allows to determine the histopathological liver lesions of different fish species (e.g. flatfish species such as dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.)). To this end, after sampling of fish, the liver should be dissected, examined macroscopically, fixed, preserved, processed histologically and stained. Then, the diagnosis and categorization of hepatic lesion types should be performed. Therefore, the main focus of the following protocol is on routine histological techniques, involving paraffin wax-embedded material, staining with hematoxylin/eosin (H&E), and examination by means of light microscopy. Finally, histopathological/histochemical changes occurring within the liver should be quantified.

Material

Equipment

- Graduated cylinders;
- Glass beakers;
- Precision balance;
- Ruler;
- Scissors;
- Forceps;
- Sharp blade (e.g. scalpel blade No. 24);
- Microtome (e.g. modern rotary microtome);
- Camera;
- Fixative containers: individual pre-labelled containers or pre-labelled histological processing cassettes;
- Stirring plate;
- Automatic tissue processors (e.g. conventional “carousel-type” processors or vacuum infiltration processors);
- Embedding moulds;
- Water bath;
- Clean glass slides;
- Adhesive (Polysine-VWR - Sigma Diagnostics);
- Hotplate;
- Synthetic mountant (e.g. D. P. X);
- Microscope.

Reagents / Solutions required

- Histological fixatives:
 - 10 % Neutral buffered formalin (NBF) - is the standard general purpose fixative; suitable for all tissues and for the subsequent application of immunohistochemical techniques and certain histochemical procedures (e.g. demonstration of lipids).

Requirements for 10 % Neutral buffered formalin (NBF) (10 litres):

Formaldehyde solution (37 % w/v) --- 1000 ml

Sodium di-hydrogen orthophosphate --- 40.0 g

Di-sodium hydrogen orthophosphate --- 65.0 g

Distilled water --- 9000 ml

- Dietrich's fixative - this fixative gives good results for liver histopathology and, in particular, it provides good nuclear fixation. Its inherent decalcifying properties are useful if other tissues are to be examined.

Requirements for Dietrich's fixative (5 litres):

Formaldehyde solution (37 % w/v) --- 500 ml

Glacial acetic acid --- 100 ml

95 % Industrial Methylated Spirit (I.M.S.) --- 1500 ml

Distilled water --- 2900 ml

- 70 % ethanol;
- Plastic embedding media (e.g. glycol methacrylate) can be used in some cases (see the reagents necessary for embedding in glycol methacrylate in the protocol described in the annex 2);
- 4 % Baker's formal saline (some cases);
- Gum sucrose solution (some cases);
- Paraffin wax;
- Industrial Methylated Spirit (I.M.S.) (70 %, 90 % and 100 %);
- Clearene or equivalent solvent;

For Haematoxylin and eosin (H&E) procedure:

- Clearing agent (e.g. "Clearene" (Surgipath, UK) or xylene);
- Graded alcohols;
- Acid/alcohol (1 % hydrochloric acid in 70 % alcohol);
- 1% aqueous Eosin Y or alcoholic eosin (5 % I.M.S.);
- Haematoxylin (Gill 3 formula) (Surgipath, UK) or equivalent.

Test procedure

1. After sampling of fish⁸, a macroscopic examination should be done.
 - 1.1 The total length of each fish should be measured prior to examination for external signs of parasitic infections and disease.
 - 1.2 Fish should be sacrificed by a blow to the head and by severing the spinal cord just posterior to the brain.
 - 1.3 The sex of the fish should also be recorded through an external or internal assessment (depending on the fish species).
 - 1.4 Remove the otoliths for age determination.
 - 1.5 Once the body cavity has been opened and the viscera exposed, the liver should first be examined *in situ* (take note of its size and colour).
 - 1.6 Then, the presence of any macroscopically visible nodules, parasites or other lesions in liver should be noted.
 - 1.7 After that, the liver should be dissected away from the remaining viscera and removed from the fish for new examination.
 - 1.8 The maximum diameter of any macroscopic nodules should be recorded as well as its general appearance, including colour, texture and degree of surface vascularization.

Note: 1) White and opaque nodules normally correspond to clear cell and acidophilic hepatocytes; 2) Yellowish coloration frequently corresponds to basophilic hepatocellular lesions; 3) Green nodules may be associated with benign and malignant tumours of the bile ducts (cholangiocarcinoma and cholangioma); and 4) Dark red staining nodules can indicate neoplasms involving vascular structures (e.g. hemangiosarcoma, hemangioma).

However, it should be noted that macroscopic appearance alone cannot be used for diagnosis.
2. The histological sampling should be the next step.
 - 2.1 The specimen should be handled with extreme care and all tissues should be placed into histological fixative or frozen in liquid nitrogen immediately after killing the fish in order to prevent the appearance of *post mortem* artefacts.
 - 2.2 If there are no grossly visible lesions, a 3 mm slice should be cut longitudinally through the central axis of the liver using a sharp blade.
 - 2.3 If visible anomalies are present, a section should also be taken through the entire depth of the affected area(s), including, where possible, adjacent normal tissue. At this juncture, take notes to describe the gross features of the lesion.
 - 2.4 Relevant photographs may also be taken to assist with the identification of small lesions during embedding.

Note: Care should be taken to avoid crushing or ripping the tissue with forceps or other dissection instruments.
 - 2.5. Two fixatives are recommended for histopathology, namely, 10 % neutral buffered formalin (NBF) and Dietrich's fixative.

⁸ Information on fish sampling procedures can be found in Feist et al. (2004).

- 2.5.1 Tissues may be placed directly into individual pre-labelled containers of fixative or into pre-labelled histological processing cassettes which allow for placing tissues from a number of fish into a common container of fixative.
- 2.5.2 Fixation is allowed to proceed for 12–24 hours with occasional agitation to ensure the even fixation of samples.
- 2.6 Samples may then be transferred to 70 % ethanol for transportation and long-term storage.
- 2.7 For some specimens, it may be desirable to embed the sample in glycol methacrylate. Note: This allows for the improved resolution in histopathology possible with thinner sections.
 - 2.7.1 For this process, tissue samples of up to 10 mm × 5 mm should be fixed in 4 % Baker's formal saline for a maximum of 24 hours.
 - 2.7.2 Then, tissues can be transferred to gum sucrose for indefinite storage at 4 °C (if required).
3. Samples of liver should be processed for embedding.
 - 3.1 For conventional histology, fixed tissue samples should be dehydrated in alcohol, cleared, and embedded in paraffin wax prior to their placement into wax blocks.
 - 3.2 Tissues should preferably be processed using commercially available automatic tissue processors (e.g. "carousel-type" tissue processors or vacuum infiltration processors). Typical histological processing schedules can be found in annex 1.
 - 3.3 After their transfer to paraffin wax (the last step in automatic processing), tissues should be embedded in wax using appropriate moulds.
 - 3.4 Plastic embedding media (e.g. glycol methacrylate) can be used for forming these blocks. Note: They have the advantage that thinner (1–2 µm) sections can be obtained, providing better resolution of histopathological features. The protocol for embedding in glycol methacrylate can be found in annex 2.
4. Then, the sectioning and staining of the liver should be done.
 - 4.1 For conventional histology, sections should be cut at 4 µm to 5 µm using a suitable microtome.
 - 4.2 The tissues should be trimmed sufficiently to allow for sectioning of the complete area of interest within the sample.

Note: Care must also be taken not to trim through any lesions that were recognized at *post mortem*. In some cases, it may be necessary to take serial sections of the sample.
 - 4.3 For general monitoring purposes, and where multiple specimens will be collected, a single section of the sample may be sufficient. However, if possible, multiple sections should be taken throughout the tissue block because they can provide greater precision in detecting focal or multifocal lesions which could be missed in a single section.
 - 4.4 Sections should be floated out in a water bath (maintained at a temperature few degrees below the melting point of the wax used to embed the specimen).

- 4.5 Sections should be then picked up on clean glass slides, which can, if necessary, be pre-coated with an suitable adhesive (Polysine-VWR – Sigma Diagnostics) to avoid the loss of sections during the staining process.
- 4.6 Drying of mounted sections should be carried out in a dust-free environment on a hotplate, following which sections should be stained with haematoxylin and eosin (H&E)⁹, dehydrated, cleared, and mounted (see annex 3 - Procedure for haematoxylin and eosin (H&E)).
5. After complete the previous steps, the histopathological diagnosis and categorization of lesion types within the liver of fish can be performed.
- Do not forget:** The examination of each section should take into account any gross observations made at *post mortem* (or necropsy).
- 5.1 Examine the slide by eye before placing it on the microscope in order to check for any large but indistinct lesions.
- 5.2 In the microscope, slides should be examined using the lowest power objective lens first.
- 5.3 Additional sections can be prepared and examined carefully in order to establish the correct diagnosis.
- 5.4 Despite non-specific lesions (e.g. inflammatory changes following bacterial, viral or parasitic infections (granulomatosis and infiltration), increased apoptosis, liquefactive and coagulative necrosis, atrophy, and the presence of increased numbers and size of “macrophage aggregates” (MAs)) are regarded as of lesser importance for environmental monitoring purposes, they should nevertheless be recorded during the assessment of liver pathology.
- 5.5 For monitoring purposes, the main lesion categories of liver (early non-neoplastic toxicopathic lesions; foci of cellular alteration (FCA); benign neoplasms and; malignant neoplasms) should be recorded.
- (Examples of these different lesion categories (and colour micrographs) can be found in Feist et al. (2004)).
6. Finally, quantify the histopathological/histochemical changes occurring within the liver.

Results

The results should be expressed as the number of histopathological/histochemical changes occurring within the liver.

References

Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A., 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish, dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences No. 38. 42 pp.

⁹ For information on special histological staining techniques for the diagnosis of liver histopathology (specific purposes) consult Feist et al. (2004).

ICES, 1998. Report of the Working Group on Pathology and Diseases of Marine Organisms. ICES CM 1998/F:4. 78 pp.

Stentiford, G.D., Longshaw, M., Lyons, B.P., Jones, G., Green, M., Feist, S.W., 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. *Mar Environ Res.* 55: 137–159.

Annexes (adapted from Feist et al., 2004)

Annexe 3. Histological processing schedules

a) Processing schedule for conventional “carousel”-type processor

Station No.	Solution
1.	70 % Industrial Methylated Spirit (I.M.S.)
2.	90 % I.M.S.
3.	90 % I.M.S.
4.	100 % I.M.S.
5.	100 % I.M.S.
6.	100 % I.M.S.
7.	100 % I.M.S./ Clearene or equivalent solvent (50:50)
8.	Clearene or equivalent solvent
9.	Clearene or equivalent solvent
10.	Paraffin wax (at 60 °C)
11.	Paraffin wax (at 60 °C)
12.	Paraffin wax (at 60 °C)

b) Processing schedule for vacuum infiltration processor (overnight processing)

Station No.	Solution
1.	70 % Industrial Methylated Spirit (I.M.S.)
2.	90 % I.M.S.
3.	100 % I.M.S.
4.	100 % I.M.S.
5.	100 % I.M.S.
6.	100 % I.M.S.
7.	Clearene solvent or equivalent
8.	Clearene solvent or equivalent
9.	Clearene solvent or equivalent
10.	Clearene solvent or equivalent
11.	Paraffin wax (at 60 °C)
12.	Paraffin wax (at 60 °C)
13.	Paraffin wax (at 60 °C)
14.	Paraffin wax (at 60 °C)

Note: Samples can be placed in 70 % I.M.S. or Gum sucrose solution before processing begins. All stations should be routinely set for one-hour duration; however this period could be reduced if necessary. Very rapid processing can be achieved if small samples are used. Moreover, it is possible to complete processing within a working day if desired.

Annexe 4. Processing schedule for embedding tissues in glycol methacrylate

Monomer-Solution:

2-Hydroxyethylmethacrylate --- 80 ml
(Merck - stabilized with 200 ppm Hydroquinone)
2-Butoxyethanol --- 12 ml
(Ethylene glycol monobutylether)
Benzoylperoxide (with 25 % H₂O) --- 0.27 g

Activator:

Polyethylene glycol 200 (Merck) --- 10 ml
N,N-Dimethylaniline --- 1 ml

Fixation: Tissue blocks (ca. 5 mm × 5 mm × 5 mm) for 24 hours (4 °C) in Baker's Formol saline, blocks should be then stored in Gum Sucrose solution at 4 °C.

Dehydration: All steps with continuous agitation.

1. Rinse twice for 15 min in distilled water or 0.1 M phosphate buffer, pH 7.4.
2. Rinse once for 30 min in distilled water or 0.1 M phosphate buffer, pH 7.4.
3. Rinse twice for 15 min in 70 % acetone.
4. Rinse twice for 15 min in 100 % acetone.
5. Rinse overnight in monomer solution.

Embedding: For each 5 ml Monomer add 0.13 ml Activator

1. Fill the embedding moulds.
2. Put in the tissue blocks.
3. Set the blockholder.

Polymerize for 4 hours (or overnight) in the refrigerator at 4 °C.

Annexe 5. Staining schedule / Procedure for haematoxylin and eosin (H&E)

Method:

Part 1: Taking slides to water

1. Place slides in Clearene to remove wax for a minimum of 2 minutes.
2. Repeat step 1 in fresh Clearene.
3. Place in 100 % alcohol to remove Clearene for a minimum of 2 minutes.
4. Repeat step 3 in fresh 100 % alcohol.
5. Wash slides in running tap water for 2–5 minutes, slides should be clear, not cloudy.

Part 2: Staining

1. Place in haematoxylin for 3 to 4 minutes.
2. Blue in running tap water for up to 10 minutes (It is not possible to “over blue”).
3. Differentiate in acid/alcohol for a maximum of 10 seconds.
4. Rinse in running tap water until blue.
5. Microscope check for clear cytoplasm and blue nuclei.
6. Place in eosin solution for 3 minutes.
7. If necessary, wash for up to 1 minute in running tap water to differentiate eosin (take care not to over differentiate).

Part 3: Dehydration, clearing, and mounting;

1. Rinse well in 70 % alcohol for 30 seconds.
2. Place in 100 % alcohol for 1-2 minutes.
3. Repeat, using fresh alcohol.
4. Place in 50/50 alcohol/Clearene for 1-2 minutes.
5. Place in Clearene for 2 minutes.
6. Repeat, using fresh Clearene.
7. Mount in a synthetic mountant such as D. P. X. and leave to dry.

Results:

Nuclei: blue

Muscle fibre: red

Red blood cells: bright red (depending on fixative)

Collagen: pink

2.2.7. Determination of micronuclei (MN) in erythrocytes of teleost fishes

Principle of the test

Micronuclei (MN) are formed by condensation of chromosomal fragments or whole chromosomes that are not included in the main nucleus following anaphase. The micronucleus assay originally developed with mammalian species can be used to evaluate the genotoxic activity of chemicals. This test in teleost fish erythrocytes (which are nucleated) has the potential for detecting clastogenic and aneugenic substances in aqueous media. Therefore, the following protocol allows to determine the MN frequency in mature erythrocytes as a genotoxicity biomarker and specifically a

measure of clastogenic and aneugenic activity. To this end, blood of fishes should be collected, smeared on ethanol-washed slides, fixed in absolute methanol, stained with Giemsa 5 % and covered with DPX mounting medium and with a coverslip. The slides should be observed under a light microscope.

Material

Equipment

- Syringe fitted with a needle;
- Clean glass slides;
- Coverslip;
- Light microscope with immersion lens.

Reagents

- Giemsa (Merck) 5 %: 5 ml Giemsa in 95 ml phosphate buffer. Filter.
Note: This solution should be previously prepared.
- Phosphate buffer 3 mM:
KH₂PO₄ - 0.375 g in 750 ml distilled water;
K₂HPO₄ - 0.2445 g in 500 ml distilled water;
Add KH₂PO₄ to K₂HPO₄ until pH 6.8.
- Ethanol 70 %;
- Absolute methanol;
- DPX mounting medium fast (Panreac).

Test procedure

1. Collect the blood from the caudal vein of fish (using a syringe fitted with a needle) and smear it on ethanol-washed microscope slide. Two slides per fish should be prepared.
2. Allow to air dry (2-5 minutes).
3. Fix the two blood smear slides in absolute methanol for 10 min.
4. Allow to air dry (2 minutes).
5. Stain with Giemsa 5 % for 30 min.
6. Remove the excess of stain with tap water and then with distilled water.
7. Allow to air dry.
8. Cover with DPX mounting medium and with a coverslip (optional).
9. The slides should be observed under a light microscope using the immersion lens.
10. Micronuclei should be recorded in 2500 erythrocytes per slide.

The criteria for scoring micronuclei are as follows:

- ✓ The size of the MN should be less than 1/3 of the main nucleus (small chromatin bodies);

- ✓ MN are circular or oval in shape, non-refractive, are on the same focal-plane and have the same colour of the main nucleus;
- ✓ Cellular membrane must not be damaged.

Results

The results should be presented as the total number of micronucleus per 2500 erythrocytes i.e. the MN frequency in mature erythrocytes.

References

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2.2.8. Assessment of lysosomal membrane stability (LMS) *in vivo* cells (mussels): neutral red retention (NRR) assay

Principle of the test

Lysosomal membrane stability (LMS) in mussels can be used as a general stress biomarker of chemical pollution as well as an integrated pathophysiological indicator of health status. This biomarker can be assessed through an *in vivo* cytochemical method (known as Neutral Red Retention (NRR) assay) based on the retention of neutral red, an amphiphilic and weak cationic dye, which freely permeates the cell membrane. Within cells the dye becomes trapped by protonisation in the lysosomes and sequestered by the lysosomal matrix, accumulating in these organelles. The degree of trapping of this lysosomotropic marker depends on the pH of the lysosome and the efficiency of its membrane associated proton pump. The NRR assay reflects the efflux of the neutral red into the cytosol following damage to the membrane and, possibly, impairment of the H⁺ ion pump. Any impairment of this latter pump will result in a reduction of the retention time (RT) of the neutral red inside the lysosomes.

The basis of NRR assay is that lysosomes in healthy cells take up and retain larger quantities of neutral red than those from damaged cells. So, in this assay, the haemolymph (blood) of mussels should be extracted and neutral red dye incubated.

Then, the progress of dye uptake into the cells as well as leakage back into the cytosol (in the case of damaged cells) should be visualized using a light microscope. Additionally, any other lysosomal abnormalities should be determined and quantified. Thus, the retention time of the neutral red by the lysosomes can be recorded.

The following NRR assay protocol should be used on living haemolymph granulocytes of mussels (e.g. *Mytilus edulis*, *Mytilus galloprovincialis*, *Mytilus trossulus*, *Perna viridis*) which are usually easy to obtain without harming the host. This NRR assay is non-destructive thus, if the test animals are not unduly stressed during collection in the field, they can be returned to their habitat after careful extraction of a haemolymph (blood) sample.

Material

Equipment

- Graduated cylinders;
- Glass beakers;
- Precision balance;
- pH meter;
- Salinity meter;
- Filter 0.45 μm ;
- Glass bottles;
- Refrigerator;
- Clean glass slides;
- Coverslip (22x22);
- Microtubes (1.5-2 ml);
- Material to keep mussel valves apart (e.g. scalpel, a pipette tip (100-1000 cc), dissection scissors);
- Hypodermic syringe of 1 ml fitted with a 21-gauge needle;
- Pipettes (1-10 μl , 10-100 μl , 100-1000 μl and 1-10 ml);
- Controlled temperature incubator (if necessary can be replaced with ice water);
- Light-proof humidity chamber for incubation of the cells with neutral red;
- Good quality bright-field binocular microscope (preferably inverted microscope but upright microscope can also be used) with $\times 10$, $\times 25$ and $\times 40$ objectives lens;
- Digital camera;
- 580 nm green filter to enhance the contrast of the neutral red (optional use).

Reagents / Solutions

- **Neutral red** - The highest strength/purity neutral red dye powder ($\geq 90\%$) should be used (if possible). Only dye batches of similar concentration and quality should be utilised when making comparisons (e.g. between sites) in a monitoring assay.
- **Filtered seawater** - The use of ambient filtered seawater (SW) (filter 0.45 μm) from the mussel sampling sites is recommended. Instead, a physiological saline

adjusted to the sampling site salinity can be prepared in the laboratory. Note: More robust inter-site comparisons can be achieved using a physiological saline with adjusted salinity because all samples are compared to a common baseline.

- **Standard mussel physiological saline formula:**

4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 4.77 g

Sodium chloride (NaCl) 25.48 g

Magnesium sulphate (MgSO₄) 13.06 g

Potassium chloride (KCl) 0.75 g

Calcium chloride (CaCl₂) 1.47 g

Dissolve the above salts in approximately 800 ml of distilled water and then make up to one litre adding more distilled water. The solution should be stored in a refrigerator, raised to room temperature prior to use, and the pH checked and adjusted to 7.36 with 1M NaOH.

CAUTION: The salinity of the above physiological saline should be approximately 30.5 PSU and thus it needs to be adjusted to the equivalent ionic strength of the ambient seawater.

- **Neutral red stock solution** - Prepare a 100 mM stock solution of neutral red by dissolving 28.8 mg of dye powder in 1 ml of dimethyl sulfoxide (DMSO) and store in the refrigerator prior to use. The stock solution will last approximately 2–3 weeks when stored in this way. Nevertheless, the solution will solidify in the refrigerator and should be raised to room temperature for dilution to the working strength stock.
- **Neutral red working solution** - For a working solution, dilute 10 µl of stock neutral red in 5 ml of physiological saline. The working solution will last about four hours before the dye begins to precipitate out.
CAUTION: Different grades of dye will have a different effect on the lysosomes depending on their purity and strength.
- **Siliconizing reagent for glass and other surfaces** (i.e. Sigmacote® from SIGMA-ALDRICH®) - This reagent retards clotting of blood or plasma and can be reusable if kept free of moisture. Use the siliconizing reagent on microtubes but only when haemolymph samples are not processed immediately after extraction. Alternatively, pre-siliconised microtubes may be used.
- **Poly-L-Lysine** (P 8920 SIGMA-ALDRICH®) (0.1 % w/v) – is a slide adhesive solution for use in adhering tissue sections to glass slides. Alternatively, pre-coated slides may be utilised. Note: Its use is recommended though it is not essential.

Test procedure

1. The haemolymph (blood) of mussels should be extracted and neutral red dye incubated through the following procedure:

1.1 First the slides and the microtubes should be prepared.

- 1.1.1 Slides should be pre-labelled and a circle should be drawn on the reverse side of the slides before use to easily identify the area on which granulocytes will be attached.
 - 1.1.2 Fill microtubes with siliconizing reagent, leave for 10-30 minutes, and then return siliconizing reagent to container. Keep microtubes on crushed ice.
 - 1.1.3 Put 2 μL of Poly-L-Lysine (0.1 % w/v, in distilled water) on the circled area of the slides and spread out with a coverslip. Leave to dry in a humidity chamber.
 - 1.2 Mussels should be prepared for the haemolymph extraction.
 - 1.2.1 The mussel valves should be carefully prised apart along the ventral surface using a solid scalpel. The scalpel should remain in position in order to keep the valves apart. The blade width of a scalpel should be enough to hold the valves apart far enough to insert a hypodermic needle. Nevertheless, other items can be used for this purpose (e.g. a pipette tip can be inserted between the open valves; or a dissection scissors that can be simultaneously used as a support¹⁰).
 - 1.2.2 Any water retained within the shell cavity should be drained out before attempting to withdraw any haemolymph.
 - 1.3 Withdraw 0.1 ml of haemolymph from the posterior adductor muscle using a 1ml hypodermic syringe fitted with a 21-gauge needle and containing 0.1 ml of filtered SW or physiological saline. Important notes:
 - a) Use a new syringe and needle for each specimen.
 - b) Do not withdraw more haemolymph than necessary during a successful “bleed”.
 - c) Failure to maintain a 50:50 ratio of haemolymph to physiological saline can result in the “clumping” together of granulocytes. This may also occur if the physiological saline temperature becomes too warm.
 - 1.4 Then, the needle should be removed from the syringe (to reduce the shearing forces that may damage the cells) and the contents of the syringe should be softly expelled into a 1.5–2.0 ml siliconised microtubes.
- Notes:
- a) If cells are used immediately after extraction, non-siliconised tubes can also be used.
 - b) If necessary cells can be kept in a refrigerator prior to use, but stored in siliconised microtubes for no longer than 20 minutes.
- 1.5 Gently invert the microtubes in order to mix the contents and then pipette 40 μl of the haemolymph/SW mixture (or haemolymph/physiological saline mixture) onto each slide, using a clean pipette tip for each sample.
 - 1.5.1 Prepare two slides per specimen.
 - 1.5.2 Cut approximately 3 mm off the end of the pipette tips prior to dispensing haemolymph onto the slides. Note: This will enlarge the tip aperture and help to decrease the potential shearing forces that can damage haemocytes during pipetting.
 - 1.5.3 Haemolymph-saline mixture should be dispensed in the same position where the Poly-L-lysine was added (circled area).
 - 1.6 Place all slides into a light-proof humidity chamber and incubate for 15-20 minutes.

¹⁰ This is useful to facilitate a steady hand during the extraction of haemolymph.

- 1.6.1 The slide preparations should be kept cool (15-18 °C) during the period of cell attachment, dye incubation and the time intervals between microscopic assessments. To this end, place the light-proof humidity chamber inside a controlled temperature incubator or place a thin layer of ice water in the dark humidity chamber. Note: The slides must not be in direct contact with the ice water and should be placed on racks to allow enough space (approximately 3 cm) for the chilled air to circulate.
- 1.7 After the incubation period (15-20 minutes), the excess suspension should be carefully drained from the slides by placing each slide on its side and letting the liquid run off.
- 1.8 Immediately pipette 40 µl of neutral red (NR) working solution onto the haemocytes and allow the penetration of neutral red in the cells.
- 1.8.1 Do not drop the neutral red working solution onto the cells; instead touch the surface of the slide with the pipette tip and slowly eject the dye onto the cells in the same position where cell suspension was added.
- 1.9 After 15 minutes, the neutral red working solution in excess should be carefully drained from the slide by placing the slide on its side and letting the liquid run off.
- 1.10 Immediately apply ambient filtered SW or physiological saline to the slide.
- 1.11 The slides can be analysed using either an upright or inverted microscope but in both cases it is necessary to apply a 22x22 coverslip.
- 1.12 One of the duplicate slides should be checked systematically under the microscope after 15, 30, 60, 90, 120, 150 and 180 minutes of incubation with NR. Note: Since neutral red is photosensitive, all slides should receive the same exposure to light under the microscope, and the light intensity should be kept as low as possible.
- 1.13 The whole circled area on the slide should be scanned and the slide replaced in the humidity chamber as soon as possible (ideally 1 minute per slide maximum).
- 1.14 Cells should be examined at each time increment for both structural abnormalities and NR probe retention time.
- 1.14.1 The retention time of the NR probe by the lysosomes should be recorded by estimating the proportion of cells displaying leakage from the lysosomes into the cytosol and/or exhibiting abnormalities in lysosomal colour and size (e.g. enlargement). Cell shape may also change as a consequence of contaminant impact.¹¹
- 1.14.2. Neutral red retention time (NRRT) will correspond to the last time period recorded when there was no evidence of dye loss or lysosomal abnormalities in more than 50 % of the cells, based on either a visual or a digital photographic determination.
- Note: In NRR assay, elongated granulocytes containing normal size lysosomes and able to retain the NR dye inside the lysosomes can be considered healthy.

¹¹ Images of the mussel granulocyte cells (haemocytes) showing uptake of neutral red in lysosomes as well as different lysosomal alteration types can be found in Martínez-Gómez et al (2015).

Caution: If the first slide does not have a sufficient number of attached haemocytes (< 200 cells) at the initial observation of 15 minutes due to a technical error, the duplicate slide can be used as a “replacement” or “back-up” slide.

Results

At each observation time using the NRR assay, two signs should be recorded:

- **Plus (+) sign:** If more than 50 % of the cells show a clear cytosol and there is no evidence of lysosomal abnormalities;
- **Negative (-) sign:** If there is evidence of dye loss and/or lysosomal abnormalities in more than 50 % of the cells.

The NRRT is the last time period when the lysosomal state is a plus sign (+). Thus, the results of this analysis should be expressed in minutes.

NOTE: The percentage of lysosomal membrane stability can also be calculated when assessing LMS at each observation time using the NRR assay. This type of data can be recorded using a score procedure (at 15, 30, 60, 90 and 120 minutes incubation for evidence of 50 % or greater of the cells exhibit no effect or different pathologies) according to Martínez-Gómez et al (2015) and as follows:

$$\% \text{ LMS} = (1 - (\text{sum of weighted score} / 75)) * 100$$

Weighted score: should be calculated by multiplying the score by the weighting factor for that time period.

References

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Chapter 3. Ecological assessment

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The ecological monitoring is essential to assess the biological effects at the population or community structure level in the event of a Hazardous and Noxious Substance (HNS) spill.

The studies should be focused on phytoplankton, benthic invertebrate fauna and intertidal rocky shore organisms (algae and invertebrates).

The methodologies applied for HNS with different behaviours in seawater (e.g. benzene (evaporator), decanoic acid (floaters/persistent), tetrachloroethylene (sinker), acrylonitrile (dissolver/evaporator)) should be based on the estimation of different parameters such as species composition, abundance and biomass that, in general, are well established as relevant indices to draw a diagnostic on the status of the communities.

At population level, since seabirds can also be affected during HNS spills, their mortality should be systematically monitored. Additionally, in most cases the survey of marine mammals' population (e.g. seals, cetaceans) should also be considered.

3.1. Determination of phytoplankton species composition, abundance and biomass

Based on HELCOM (2017)

Principle of the test

This protocol allows to determine the species composition, abundance and biomass of phytoplankton. Such parameters should be determined by counting phytoplankton from preserved water samples using the Utermöhl inverted light microscopical method (Utermöhl, 1958).

Material

Equipment

- Open container;
- Refrigerator;
- Tightly sealed glass bottles - e.g. use clear, colourless iodine-proof (i.e. glass) bottles with tightly fitting screw caps for iodine-preserved material (Note: with this bottles it is easy to see when the iodine becomes depleted and more preservative needs to be added);
- Sedimentation chambers;
- Moistened tissue paper or e.g. a small flask of water or a petri dish filled with water;
- Plastic box;

- Inverted light microscope;
- Different objectives (e.g. objective with intermediate magnification, 20-25x);
- Ocular grid of 5 x 5 (or 10 x 10) squares (helpful to count dense fields of small cells);
- Fluorescence microscopy or flow cytometry (for picoplankton counts).

Preservatives

Acid Lugol's solution (most suitable);

Alkaline Lugol's solution (if coccolithophorids need to be preserved with the coccoliths intact);

Neutral Lugol's solution (if the thecal plate pattern of dinoflagellates needs to be investigated);

Neutralised formaldehyde (only at a few coastal stations where long time series are already established using formaldehyde).

Preparation of preservatives / Reagents

Acid Lugol's solution (Willén, 1962):

200 cm³ of distilled or deionized water

20 g of potassium iodide (KI)

10 g of resublimated iodine (I₂)

20 cm³ of glacial acetic acid (conc. CH₃COOH)

1. Mix the ingredients in the order listed.
2. Make sure that the previous ingredient has dissolved completely before adding the next.
3. Store in a tightly sealed glass bottle cooled and in the dark.

Alkaline Lugol's solution (modified after Utermöhl, 1958):

1. Replace the acetic acid of the acid solution by 50 g of sodium acetate (CH₃COONa).
2. Use a small part of the water to dissolve the acetate.

Neutral Lugol's solution (from Andersen and Throndsen, 2003):

1. Prepare as acid Lugol's solution, but without the glacial acetic acid.

Other reagents

Sodium thiosulphate (Na₂S₂O₃.5 H₂O)

Test procedure

1. Net samples to be studied alive can be kept fresh for a few hours in an open container in a refrigerator.

2. All other samples should be preserved in glass bottles immediately after sampling¹²:
 - 2.1 Use 0.25-0.5 cm³ of acid Lugol's solution per 100 cm³ of water sample.
 - 2.2 Parallel subsamples for investigating coccolithophorids or dinoflagellates should be fixed with 0.25-0.5 cm³ of alkaline or neutral Lugol's solution, respectively, per 100 cm³ of sample.
 - 2.3 If the cells are too strongly stained by iodine for comfortable identification, surplus iodine can be chemically reduced to iodide by dissolving a small amount of sodium thiosulphate (Na₂S₂O₃·5 H₂O) in the aliquot to be sedimented.
3. Preserved samples should be stored in dark and cool conditions and counted as soon as possible (at least within a year).
4. Before counting, the samples should be adapted to room temperature and then the contents of the bottles should be homogenized (shake firmly but gently in irregular jerks). Note: If a sample must be shaken vigorously in order to disperse tenacious clumps, this should not be done later than one hour before starting sedimentation.
5. Immediately after homogenization, each sample should be poured into a sedimentation chamber¹³ (placed on a level and horizontal surface (vibration free)) that should not be exposed to temperature changes.
6. The settling chambers and a moistened tissue paper or e.g. a small flask of water should be covered with an overturned plastic box (until start counting).
7. The settling times given in Table 1 are recommended as minimum times.

Table 1. Settling time for phytoplankton samples preserved with Lugol's solution for sedimentation chambers of different volumes (HELCOM, 2017).

Volume of chamber (cm ³)	Height of chamber (cm)	Settling time (h)
2-3	0.5-1	3
10	2	8
25	5	18
50	10	24

Note: Sedimentation chambers of 100 cm³ (height 20 cm and settling time 48h for Lugol's solution) should be used only when phytoplankton is very sparse.

8. Phytoplankton counting procedure using an inverted microscope should be performed within four days:
 - 8.1 First, the sedimented sample should be examined for general distribution of cells on the chamber bottom (placed in the microscope), as well as the abundance and size distribution of the organisms. The settled sample should be discarded if the distribution of cells is visibly uneven.
 - 8.2 Then, start counting at the lowest magnification followed by analysis at successively higher magnifications (or the other way around, start with the highest magnification).

¹² Information on phytoplankton sampling (methods and equipment) can be found in HELCOM (2017).

¹³ Clean carefully the sedimentation chamber after use (Do not let it dry before cleaning!)

- 8.3 Try to keep the same magnification to count the specific species and if necessary decrease the volume settled or the area counted if a species is very abundant.
- 8.4 Large, easily identifiable species, which are generally relatively sparse, should be counted at the lowest magnification and preferably over the entire chamber bottom.
- 8.5 Smaller species should be counted at higher magnification and possibly only on a part of the chamber bottom.
- 8.6 The recommended magnifications for phytoplankton of different sizes are listed in Table 2.

Table 2. Recommended magnifications for counting of different size classes of phytoplankton (HELCOM, 2017).

Size class	Magnification
0.2-2 μm (picoplankton)*	1000x
2-20 μm (nanoplankton)	200-630x
>20 μm (microplankton)**	100-250x

* Picoplankton cannot be properly analysed using the Utermöhl method; requires fluorescence microscopy or flow cytometry (see OSPAR, 2016).

** Small microplankton species can preferably be counted together with the nanoplankton when they are abundant or they can be counted using an objective with intermediate magnification (20-25x).

- 8.7 Counting the whole chamber bottom should be performed by traversing back and forth (or up and down) across the chamber bottom. The parallel eyepiece threads delimit the transect where the phytoplankton should be counted (Fig. 1.). Count the phytoplankton cells crossing the upper thread, but not those crossing the lower thread.

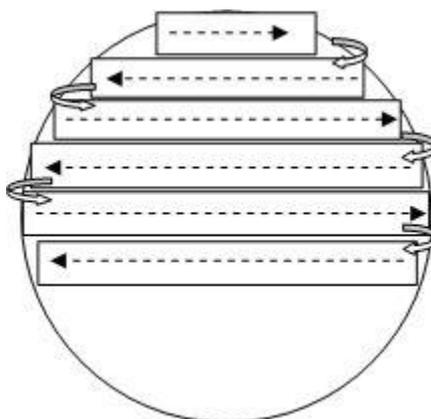


Figure 1. Traversing the whole chamber bottom with the parallel eyepiece threads to indicate the counted area (Edler and Elbrächter, 2010).

- 8.8 If half the chamber bottom is to be analysed, every second transect of the whole chamber should be counted.

- 8.9 If a smaller part is to be assessed, one, two, three or more diameter transects should be counted.
- 8.10 Rotate the chamber 30-45° after each transect be counted.
- 8.11 A number of fields-of-view, or ocular grids of 10 x 10 squares, can also be counted.
- 8.12 If ocular squares (grids) are used in counting:
- 8.12.1 Single cells crossing two sides of the square (e.g. the bottom and the right sides of the square) should be counted, and cells crossing the other two sides (e.g. the left and the upper sides of the square) should be ignored (Fig. 2a).
- 8.12.2 In the case of filamentous and colonial species, those cells of the filaments and colonies that occur inside the square should be counted (irrespective of which side of the square the filament or colony crosses). However, the cells of the same filaments and colonies occurring outside the square should not be counted (Fig. 2b).

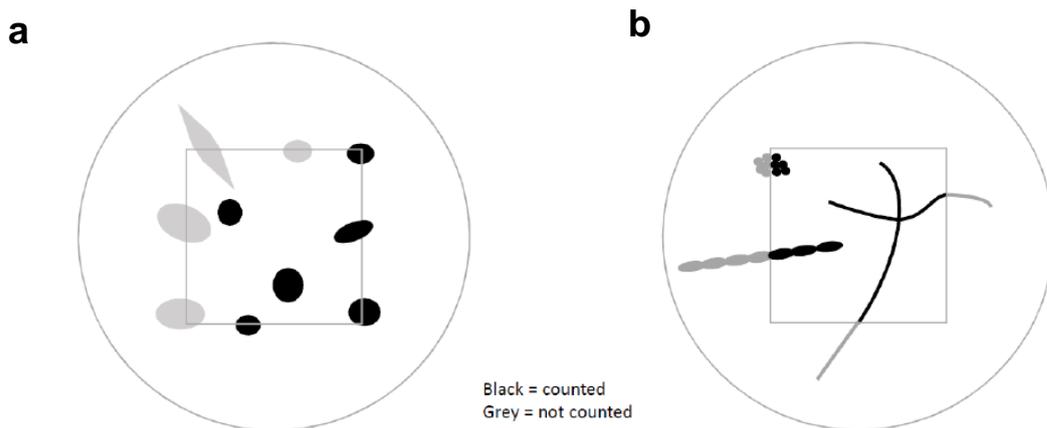


Figure 2. How to count a) single cells and cenobias; b) filaments and colonies (HELCOM, 2017).

Observations – counting units

- The common counting unit is the cell. This applies also to colonies with irregular numbers of cells. Cell numbers in densely-packed and small-celled colonies may be estimated by 1) visual dividing of the colony into sub-areas; 2) counting cell numbers in one sub-area and; 3) multiplying with the number of sub-areas.
Note: For estimate the total cell number of a colony, it is essential to take into account if the colony is hollow or filled with cells as well as its potential three-dimensionality.
- Filamentous cyanobacteria should be counted in lengths of 100 µm. Numbers of 100 µm pieces per volume of seawater should be reported.
- Diatoms with any plasma inside the cell should be counted as a living cell.

- The mixotrophic ciliates *Mesodinium rubrum* and *Laboea* spp. (protozooplankton) should be counted and included in abundance and biomass values of phytoplankton.
- At least 50 counting units of each dominating taxon should be counted, and the total count should exceed 500 units. All cells encountered in the area examined should be counted and reported even if fewer counted units progressively will decrease the precision of the count and increase the statistical error of the population estimate.

Results

The approximate 95 % confidence limits of a number of counted units can be calculated from the following formula:

$$95 \% \text{ C.L.} = n \pm 2 \times (100/\sqrt{n}) \%$$

where n is the number of units counted.

Phytoplankton abundance results are given as counting units per volume of seawater:

The number of counting units per volume (dm^3) of seawater should be calculated by multiplying the number of units counted with the coefficient C, which can be obtained through the following formulas:

$$C(\text{dm}^{-3}) = A * 1000 / (N * a_1 * V) \text{ or}$$

$$C(\text{dm}^{-3}) = A * 1000 / (a_2 * V)$$

where:

A = cross-section area of the top cylinder of the combined sedimentation chamber; the usual inner diameter is 25.0 mm, giving $A = 491 \text{ mm}^2$ (the inner diameter of the bottom-plate being irrelevant)

N = number of counted fields or transects

a_1 = area of single field or transect

a_2 = total counted area

V = volume (cm^3) of sedimented aliquot

Phytoplankton biomass can be expressed as cell volume (or weight):

- For a reliable biovolume calculation, the species (individuals) have to be allocated to size classes during the counting process. The individual biovolumes of the different counting units have to be multiplied with their abundance to get the biovolume per dm^3 :

$$\text{Biovolume}_{\text{taxon}} [\text{mm}^3 \text{ dm}^{-3}] = \text{abundance} [\text{dm}^{-3}] \times \text{VCU} \times 10^{-9}$$

VCU = volume of counting unit (in μm^3)

- Biomass (wet weight) can be simply derived from the biovolume data by a rough assumption of a plasma density of 1 g cm^{-3} , as follows (CEN, 2015):

$1 \text{ mm}^3 \text{ l}^{-1}$ (biovolume) = $1 \text{ cm}^3 \text{ m}^{-3}$ (biovolume) = 1 mg l^{-1} (wet weight):

$1 \text{ mm}^3 \text{ m}^{-3}$ (biovolume) = $10^6 \mu\text{m}^3 \text{ l}^{-1}$ (biovolume) = $1 \mu\text{g l}^{-1}$ (wet weight)

Quality control of methods

- Extensive knowledge of the taxonomy, identification and counting procedures of phytoplankton is crucial in order to produce high-quality data. Therefore, the analyst has to be skilled.
- It is recommended to count one dominating species using a low and one using a high magnification in a new subsample in every 20th sample in order to check the precision of the analyst and method.

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3.2. Determination of the species composition, abundance and biomass of benthic invertebrate fauna

Based on Rumohr (2009)

Principle of the test

This protocol allows to determine the species composition, abundance and biomass of benthic invertebrate fauna. Such parameters should be determined firstly through a separation of fauna from the sediment by a sieving procedure. The material retained on the sieves should be fixed, stained (some cases), sorted and counted. If it is impossible to sieve the material before fixation, the fixed material can be sieved. Biomass can be measured through wet weight, dry weight, and/or ash-free dry weight, either from fresh or fixed material.

Material

Equipment

- Containers;
- Water sprinklers;
- Sieves:
 - Larger;
 - Mesh size of 1.0 mm;
 - Mesh size of 0.5 mm or even finer;
 - Mesh size finer than that of the initial sieve (if resieving of samples is necessary);
 - Integrated sieve tables can be used.
- PH meter;
- Magnification lamp;
- Stereomicroscope;
- Sorting aid (e.g. a “fluidized sand bath”, see Holme and McIntyre, 1984);
- PVC trough (5 m long, 20 cm wide, 20 cm high);
- Ordinary rain gutter with 5 m of length (with one open and one closed end);
- Different types of sample splitter can be used;
- Precision balance;
- Filter paper;
- Puncture needle;
- Freeze-drier;
- Oven;
- Muffle furnace;
- Desiccator.

Reagents

- Formaldehyde solution (40 %) ¹⁴;
- Buffered 4 % formaldehyde solution (1 part 40 % formaldehyde solution and 9 parts filtered seawater); For buffering, 100 g of hexamethylene tetramine (Hexamine, Urotropine) can be used per 1 l of concentrated formaldehyde (36-40 %). Sodium tetraborate (Borax) in excess can also be used.
- Absolute ethyl alcohol;
- Phenol;
- Alkaline (pH = 9) fluid.

Stains

- Rose Bengal;
- Eosin;
- Other stain.

Test procedure

1. Fauna should be separated from the sediment through a sieving procedure, as follows:
 - 1.1 The grab or boxcore (samplers) ¹⁵ should be emptied into a container, and then the sample should be transferred, portion by portion, onto the sieves as a sediment-water suspension.
 - 1.2 Sprinklers or hand-operated douches can be used to suspend the sample.
 - 1.3 Very stiff clay can be gently fragmented by hand in the water of the container.
 - 1.4 The sieve must be cleaned after each portion has been sieved.
 - 1.5 The sieve should be agitated gently under the water surface of a water-filled container, until all sediment that can pass the sieve has been washed through.
 - 1.6 Pick out by hand fragile animals (e.g. some polychaetes), stones and large shells during the sieving.
 - 1.7 All the material retained on the sieve should be carefully flushed off the sieve into an appropriate container with water from below (avoid the use of spoons or other scraping tools).

Note: If a 0.5 mm sieve is used, the 0.5 mm and the 1 mm fractions must be kept separate throughout all further processing.
2. The material retained on the sieves should be fixed in a buffered 4 % formaldehyde solution (Note: Sponges are best preserved by putting them directly into absolute ethyl alcohol).
3. For animal sorting, the fixed samples must first be thoroughly washed with tap water and left to soak overnight, thus avoiding the exposure to formalin vapour.
4. The samples can be stained ¹⁶ in order to facilitate sorting and to increase sorting accuracy, especially for small animals:

¹⁴ Formaldehyde is a toxic compound; known as a carcinogen.

¹⁵ Information on benthic invertebrate fauna sampling (methods and equipment) can be found in Rumohr (2009).

¹⁶ In some cases, staining could cause problems with species identification.

- 4.1 Wash the sample free from the preservation or fixation fluid using a sieve with a mesh size smaller than 0.5×0.5 mm.
 - 4.2 Allow the sieve to stand in Rose Bengal or other stain (1 g l^{-1} of tap water plus 5 g of phenol for adjustment to pH=4-5) for 20 min with the sample well covered.
 - 4.3 Wash the sample until the tap water is no longer coloured.
 - 4.4 As an alternative, Rose Bengal (4 g l^{-1} of 40 % formaldehyde) or Eosin could be added to the fixation fluid.
 - 4.5 Overstained specimens can be destained in alkaline (pH=9) fluids.
 5. If it is impossible to sieve the material before fixation, the fixed material can be sieved.
- Note: The sorting characteristics of fixed material are different from those of live fauna and result in apparently greater abundance and biomass figures.
6. Sorting must be done using a magnification aid such as a magnification lamp or a stereomicroscope. Any finer fraction (<1 mm) should always be sorted under a stereomicroscope.
 7. To reduce sorting time, a sorting aid (e.g. a “fluidized sand bath”) could be used, if its efficiency is satisfactorily checked for the bottom material being sorted.
 8. The Ludox method (see Higgins and Thiel, 1988) can also be useful for the extraction of soft-bodied macrofauna.
 9. For coarse sand: i) The sediment sample should be placed on a PVC trough; ii) An ordinary rain gutter can be used; iii) Water should be poured over the sediment from one closed end and the extracted fauna should be caught on a sieve on the other (open) end; d) The residue should be checked for larger (heavier) animals.
 10. If taxa are present in large numbers (e.g. *Polydora*, capitellids, phoronids), it might be advisable to split the (entire) sample to reduce the counting time. Otherwise, avoid sample splitting! Rare species should be counted using whole samples.
 11. Taxa should be determined to the lowest possible level.
 12. Fragments should be kept for biomass determinations, but only heads of partial organisms (e.g. polychaetes) should be counted.
 13. Encrusting or colonial species should be mentioned (presence/absence) and their abundance can be reported in a five-level, semi-quantitative code (none-single-few-many-large number).
 14. Biomass can be measured through wet weight, dry weight, and/or ash-free dry weight, either from fresh or fixed material.

14.1 Wet weigh:

Note: Fresh wet weight should be preferred to formalin-wet weight, but if the latter has to be used, weighing should not be performed until at least three months after fixation.

i. First, remove the excess fluid using filter paper:

- Animals should be left on the filter paper until no more distinct wet traces can be seen.
- The water should be drained off bivalves;
- Echinoids should be punctured to drain off the water before blotting on filter paper.

ii. Then, the organisms should be weighed with the accuracy required (e.g. for adult macrofauna, ± 0.1 mg):

- Animals with shells should be weighed with their shells.
- In case tube-building animals have to be weighed together with their tubes, appropriate correction factors should be established.

14.2 **Dry weight** should be estimated after drying the fresh material at 60 °C, or by freeze-drying, until constant weight is reached. This will take at least 12-24 h, depending on the thickness of the material; large bivalves might require up to 96 h.

14.3 **Ash-free dry weight** (most accurate measure of biomass but it destroys specimens) should be determined after measuring dry weight. Samples should be incinerated at 500 °C in a muffle furnace (approximately 6 h, depending on sample type and size) until constant weight is reached (Note: Avoid exceeding 550 °C!). Before weighing the incinerated material, the samples must be kept in a desiccator, while cooling down to room temperature.

Results

- If different sieves are used (a sieve with a mesh size of 1.0 mm and a finer sieve), the results should be given for the individual and the summed fractions.
- Results should include data for individual samples (each grab and boxcore sample) and/or average values per m² with standard errors or standard deviations and number of samples, for both abundance and biomass for each taxon and the total fauna.
- Final values should be expressed as number of individuals (for abundance results) and grams (g) (for biomass results).

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3.3. Determination of the species composition and abundance on marine intertidal rocky shores

Based on Hawkins and Jones (1992) and Cabral-Oliveira (2013)

Principle of the test

This protocol allows to determine the species composition and abundance (of macroalgae and invertebrate macrofauna) on marine intertidal rocky shores. Such parameters should be determined through a non-destructive sampling (visual census) considering all intertidal zonation levels (littoral fringe, eulittoral and sublittoral fringe). Randomly-located quadrats (50 x 50 cm) should be sampled in three sites (in each location to be analysed). The survey is carried out in the field by estimation of the percent cover (for algae) and number of individuals (for invertebrates) found in the quadrat.

Material

- Quadrats with different sizes (50 x 50 cm, 10 x 10 cm); each quadrat can be divided into 25 sub-quadrats, to help quantify the percent cover.
- Piece of string or tape measure;
- Camera (to take pictures of each square if desired);
- Waterproof notebook for pointing values.

Test procedure

1. A non-destructive sampling (visual census)¹⁷ should be performed in the field considering all intertidal zonation levels - littoral fringe, eulittoral and sublittoral fringe).
2. In all study areas, three sites (approximately 100 to 300 m apart) should be selected in each location.
3. Five randomly-located quadrats (50 x 50 cm) should be sampled at each site.
 - 3.1 The best way is to use random distances either side of the transect line.
 - 3.2 Sometimes it is worth marking random distances with a piece of string or tape measure before starting.
 - 3.3 If the quadrat hits a rock pool or a larger crevice reject it completely and take the next random quadrat that lands, or is placed, on open rock.
 - 3.4 In some cases, smaller quadrats (10 x 10 cm) can be used to sub-sample the larger ones. Note: For smaller organisms such as amphipods, these smaller quadrats should be used and the organisms should be collected, sorted and identified in the laboratory.
4. For each quadrat, the percent cover values of algae should be estimated through a sub-division of the quadrat and the total number of animals (e.g. limpets) should be counted in the field.
5. The identification of organisms should be done in the field whenever possible (and at the lowest taxonomic level possible). In case of taxonomic uncertainty the specimen should be collected and identified in the laboratory.¹⁸

¹⁷ Information on sampling of intertidal rocky shore organisms can be found in Hawkins and Jones (1992) and Cabral-Oliveira (2013).

¹⁸ Taxonomic expertise is necessary.

Results

The percent cover of algae filling less than 1/4 square can be set at an arbitrary value of 0.5 % (Dethier et al. 1993).

Final values should be expressed as percentages (%) (for algae) and number of individuals (for invertebrates).

Acknowledgment: We would like to thank Joana Cabral-Oliveira for reviewing this protocol.

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