Workshop 5.10
Ecotoxicity – applying the 3Rs

Applying the Three Rs in Acute Ecotoxicity

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Summary
For hazard identification/classification in Environmental Risk Assessment, the first step is to determine the acute ecotoxicity and, on basis of this, to establish a No Observed Effect Concentration (NOEC). Acute ecotoxicity is based on LC50 tests with single species. For a rough estimation of toxicity for classification and labelling purposes killing a large number of vertebrates is not justified. This paper analyses the present situation and solutions proposed to avoid or to reduce vertebrate tests either with more rational testing strategies and/or by developing animal alternatives that can provide data on this first step in the estimation of toxicity.

Keywords: ecotoxicity, three Rs, fish cells, fish embryo, testing strategies

Introduction

The assessment of the adverse effects of chemicals and other stressors on the environment is a difficult task because of the complex network of species and physicochemical factors interrelated in a given ecosystem. Since it is almost impossible to perform tests in complete ecosystems, ecotoxicity testing has to rely on simplified testing systems, but should still be as representative for the whole ecosystem as possible.

The classical approach for Environmental Risk Assessment (ERA) combines calculations of exposure, hazard identification and risk characterisation. Regulatory testing guidelines identify two environmental compartments: terrestrial and aquatic, depending on the exposure scenario.

For hazard identification/classification, the first step is to determine the acute ecotoxicity and then, based on this, the Predicted No Effect Concentration (PNEC) is established. Acute ecotoxicity is based on LC_{50} tests with single species, usually one species per trophic level (aquatic and terrestrial primary producers, invertebrates and vertebrates).

Single species LC_{50} data have been required for more than 30 years in the regulatory guidelines (EC, 1967), although their value is highly questionable with respect to toxicological relevance. To base environmental risk assessment on the death of individuals after short-term exposure to high toxicant concentrations is even more questionable – perhaps with the exception of accidental spills – but is still what we have in hand and what is accepted by the regulatory community.

Today there is a strong political ambition to reduce animal experiments and replace them with alternatives. For a rough estimation of toxicity for classification and labelling purposes it is not justified to kill a large number of vertebrates. Alternatives in ecotoxicology focus on replacement of tests performed with vertebrates. Currently, acute LC_{50} data on vertebrates are required in ERA of chemicals, active substances of plant protection products (PPP), biocides and human and veterinary medicines (EC, 1991; 1992; 1998).

Serious ethical concern has been raised about the use of vertebrates in ERA, since they suffer severe distress and pain when subjected to acutely toxic concentrations of chemicals, which is clearly not acceptable in view of animal welfare. Therefore, it is becoming more and more urgent to find solutions to avoid, or in the worst case to reduce, vertebrate testing, either with more rational testing strategies and/or by developing animal alternatives that can provide data on this first step in estimating toxicity.

Terrestrial vertebrates

Acute oral toxicity data on mammals and birds - as representatives of terrestrial vertebrate wildlife - are required for the registration of active substances (EC, 1991). 3R approaches for mammals in ERA benefit from the process of hazard characterisation for humans (OECD 2001: TG-420, 423, 425). In practice, the elaboration of a testing scheme for wild mammals is generally not necessary, as a great number of laboratory studies with mammals are carried out for the assessment of the risk to humans. Thus, data for mammals on acute, subacute, chronic toxicity and carcinogenicity, as well as findings concerning influences on reproduction and embryonic development are available for each compound. That same data can be used to assess the ecotoxicological risk for free-living mammals. Other than in special circumstances, further toxicological studies on wild mammals would generally not provide additional information that justifies the effort required and the sacrifice of animals involved.

On the other hand and in general, less attention has been paid to finding alternatives for toxicity tests on birds and those developed are limited to reduction and refinement, and refer only to acute tests. The development of new avian toxicity guidelines and updating of existing OECD guidelines was the subject of a joint SETAC/OECD workshop on Avian Toxicity Testing held in December 1994 at Pensacola (USA). Among the recommendations of the OECD working group created after the workshop, a new guideline for avian acute oral toxicity was proposed. (OECD TG-223, October 2002). At present, the guideline is being finalised after the period for public comments ended in February 2003. The new guideline favours determination of only the lethal threshold and non-observed-effect-doses, with a view to reducing the number of animals needed per test. The new guideline provides a sequential testing procedure that optimises the doses and matches the precision of the endpoint with the precision required for hazard assessment and labelling.
(http://www.oecd.org/document/62/0,2340,fr_2649_201185_2348862_1_1_1_1,00.html)

Aquatic vertebrates

While acute toxicity data on mammals and birds are only required for the registration of active substances under EU directive 91/414, acute fish bioassays need to be performed for all cited substances and chemicals, and in some EU countries, for Whole Effluent Assessment.

In the European Union, data requirements for the notification of new substances are listed in the annexes to the Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (EC, 1967). The quality and amount of data required primarily depends on the amount of the substance to be marketed (EC, 1992). The "base set" data are required for all substances for which the marketing quantity exceeds one ton per year per manufacturer (Annex VII.A of the Directive). The data reported are used to decide on the classification and labelling as well as for hazard and risk assessment of the substance. This "base set" includes acute toxicity for freshwater fish (96 h LC_{50}; OECD TG 203), acute toxicity for daphnids (48 h LC_{50}) and growth inhibition test on freshwater algae (growth rate: 72 h ECr50 and/or biomass: 72 h ECb50). The toxicity value for the most sensitive of these three organisms is used when deciding on classification and labelling as well as for hazard and risk assessment (calculation of Predicted No Effect Concentration – PNEC).

Reduction

The high demand for fish acute data has obviously promoted the interest in finding alternative approaches. Applying the 3Rs philosophy, the biggest advancement in the last years lay in the refinement of acute fish bioassays. The numbers of fish used can be reduced by 60-70% by applying a strategy proposed by Hutchinson and co-workers in 2003 based on the concept of a threshold approach (Hutchinson et al., 2003).

The principle of the threshold approach is based on findings from a number of studies (Weyers et al., 2000; Hutchinson et al., 2003, Jeram et al., 2005) that report that either Daphnia or algae are the most sensitive for approximately 80% of substances tested. Consequently, most of the current risk calculations from base set testing are based on Daphnia and algae data and fish toxicity data are used only for 20% of the substances. For 80% of the tested chemicals the fish LC_{50} test was a wasted effort. In the strategy proposed by Hutchinson et al. (2003), tests on algae and daphnia are performed first, the results are compared, and the lowest value is set as the upper threshold concentration (UTC). Using this concentration, a fish test is carried out. If no toxicity is observed, no further tests are carried out, and the acute fish toxicity result (LC_{50}) is reported as greater than (>) the UTC value. In case the fish show a toxic response, a second test is performed at a lower concentration using a dilution factor of 3.2. The testing continues by stepping down concentrations until no toxicity is observed. The 96h-LC_{50} value can be obtained from all step-down threshold test data. This approach was presented at the 23rd ESAC scientific meeting. A statement was unanimously endorsed on 28 September 2005 supporting the strategy in general. However, ESAC did not approve the step-down procedure, i.e. when fish mortality occurs in the threshold test at the UTC. For the time being, ESAC suggests that a full LC_{50} test should be performed corresponding to the requirements of OECD Test Guideline 203. (http://ecvam.jrc.cec.eu.int/index.htm).

Replacement

Replacing the acute bioassay on adult fish by adopting the fish embryo test (Nagel, 2002) was already proposed and in some countries adopted for WEA.

The rationale for this proposal is based on the animal definition in the Council Directive 86/609/EEC (EC1986; 2003): *'animal' unless otherwise qualified, means any live non-human*

vertebrate, including free-living larval and/or reproducing larval forms, but excluding foetal or embryonic forms".

In 2001, a working group convened by the German Standardisation Organisation (DIN) designed a protocol for an alternative test based on zebrafish embryos as a replacement for the acute fish test in routine whole-effluent testing (DIN, 2001). After an exposure period of 48 h after fertilisation, the toxicological endpoints coagulation, failure to develop somites, lack of tail detachment from the yolk as well as lack of heartbeat are assessed. Since zebrafish only hatch after approx. 72-96 h, this alternative method does not represent an animal test in legal terms. Since the 1st of January 2005, the so-called "zebrafish egg test" has become mandatory in Germany.

The German Federal Environment Agency has submitted a modified protocol for sewage testing to ISO and will submit another modified protocol for chemical testing to the OECD in late 2005, which will not only cover zebrafish, but also other common OECD fish species such as the fathead minnow (Pimephales promelas) and the Japanese medaka (Oryzias latipes). The proposal has been submitted to ECVAM and is on the way to be peer reviewed by ESAC. Since there is a strong commitment to alternative methods in future chemical testing within the framework of REACH (Registration, Evaluation and Authorisation of Chemicals), the new EU chemicals policy (EC, 2001), the fish embryo test seems a very promising candidate for future toxicological routine testing (Braunbeck et al., 2005; Lange et al., 1995; Nagel, 2002).

The use of embryos instead of adult fish represents a considerable advance, however, the use of fish embryos still requires the maintenance of fish facilities, and for some scientific sectors tests with embryos represent a refinement rather than a full replacement of vertebrate testing.

On the way to fully replacing fish individuals, a cytotoxicity test with fish-derived cells seems to be a realistic alternative.

In vitro assays based on fish cell lines have been used in ecotoxicology for screening, for toxicity ranking of chemicals, chemical mixtures, environmental samples and in Toxicity Identification Evaluations (T.I.E.) during the last 30 years (for review, see Castaño et al., 2003). A number of *in vitro* cytotoxicity assays using fish cells have been developed, the majority of them employing cells from salmonid and cyprinid species. As a general rule, good correlations are found among different cell lines, endpoints, and among different laboratories. In addition, there is a good correlation between the *in vitro* data and the *in vivo* LC_{50} fish data in ranking the toxicity. However, when considering sensitivity, it is clear that *in vitro* cell assays have a markedly lower sensitivity than the *in vivo* fish LC_{50} test. On average, the absolute sensitivity is one or two orders of magnitudes less than that of the acute lethality *in vivo* assays (Segner and Lenz, 1993; Castaño et al., 2003). An advantage is that *in vitro* tests do not carry the risk of indicating false positives, but the problem is that they can indicate false negatives (Castaño et al., 2003). The well recognised good correlation between *in vivo* and *in vitro* fish data favours the use of fish cells, although the lack of sensitivity and the appropriate prediction models preclude their general acceptance for testing purposes.

Regarding the sensitivity we should not forget the wide range of sensitivity found among the *in vivo* fish bioassays. As indicated before, the acute fish test has long been a mandatory component in initial toxicity testing. As a consequence, a considerable body of information has been accumulated for existing chemicals. A closer inspection of existing acute fish LC_{50} data, however, reveals differences in orders of magnitude not only between species, but also for the same species between laboratories (e.g. Juhnke and Lüdemann, 1978). On the other hand, and comparing the fish embryo test and cytotoxicity test, for some chemicals there is some evidence that the fish embryo test is slightly more sensitive and more accurately mirrors toxicity to fish than the cytotoxicity tests (Lange et al., 1995; Nagel, 2002). However, it should be noted that for particular substances cytotoxicity tests may be more sensitive than fish embryo tests (e.g., Zabel and Peterson, 1996).

Recent initiatives suggest an approach combining the 3Rs, i.e. reducing using the threshold approach, replacing (refining) by means of a fish-embryo test and replacing with fish cells (Braunbeck et al., in preparation).

Fish *systems* cannot be deleted from the initial "base set" for testing aquatic ecotoxicity, because for between 20 and 40% of the test substances fish represent the most sensitive taxon

Tab. 1: Linear correlations among EC_{50} values from different basal cytotoxicity tests performed in the RTG-2 and R1 fish cell lines and EC_{50} from two *Daphnia magna* assays for a total of 26 chemicals from the Multicenter Evaluation of *In Vitro* Cytotoxicity test (MEIC) study.

MEIC test number	43	44	45	246	247	248
43	1.00	0.99	0.98	0.98	0.96	0.96
44	0.99	1.00	0.99	0.97	0.95	0.96
45	0.98	0.99	1.00	0.97	0.96	0.96
246	0.98	0.97	0.97	1.00	0.99	0.99
247	0.96	0.95	0.96	0.99	1.00	1.00
248	0.96	0.96	0.96	0.99	1.00	1.00
53	0.86	0.85	0.84	0.88	0.90	0.90
54	0.87	0.85	0.85	0.87	0.90	0.90

Test numbers: 43, 44 and 45 = basal cytotoxicity in the R1 fish cell line; 246, 247 and 248 = basal cytotoxicity in RTG-2 fish cell line, 53 and 54 = *Daphnia magna* immobilisation assays (data taken from Clemedson et al., 1998).

(Hutchinson et al., 2003; Jeram et al., 2005). The question is how to recognise those chemicals without performing an *in vivo* fish test. The easiest way is to perform, as a first step, a cytotoxicity test with fish cell lines in order to account for potentially fish-specific effects.

Comparisons between the Daphnia test and fish cytotoxicity test have shown good correlations but differences in sensitivity for both chemicals (see tab. 1, from Clemedson et al 1998) and complex mixtures (see tab. 2, fig. 1). While correlations among EC_{50} from different fish cell lines, cytotoxicity endpoints and within different laboratories involved in the MEIC study were always $r \geq 0.96$, correlations in EC_{50} from Daphnia and EC_{50} from fish cell lines laid on average at 0.86. Therefore, *a priori*, fish cells could be able to recognise toxic compounds that Daphnia does not recognise and *vice versa*. However at the present we cannot determine whether those are fish-specific toxic chemicals, because most chemicals used in the MEIC study were not environmentally relevant and it is difficult to find *in vivo* LC_{50} data on fish. As soon as a fish cytotoxicity test is fully validated and the correction or prediction factor established, it can readily be used as a truly alternative method to identify those substances for which fish are likely to be more sensitive than daphnids or algae.

In cases when there is uncertainty whether fish cytotoxicity can be predicted accurately, the fish test LC_{50} (OECD TG-203) or, if properly validated, the fish embryo test could be applied to make an *in vivo* comparison. Alternatively, the cytotoxicity EC_{50} value (after being corrected by the appropriate prediction model) could indicate whether the sensitivity of fish systems is lower than that of algae or daphnids. This "negative result" indicates that the test substance is not specifically toxic to fish, and no further testing would be required, since subsequent risk assessment procedures would be based on Daphnia or algae EC_{50} values. The proposal ensures that any testing with adult fish will be reduced to the minimum and if embryo fish were fully validated, testing with adult fish would be avoided, and it reduces the number of tests to be carried out with fish embryos to the absolute minimum.

Tab. 2: Comparison between EC_{50} from *D. magna* inmobilisation test and EC_{50} from Neutral Red Uptake on the RTG-2 fish cell line after testing 49 samples of complex mixtures (industrial effluents). 92% of the effluents are toxic for both test. 2/3 correlates positively (r=0.62, p<0.001) (fig 1). 1/3 do not correlates at all. The conclusion is that complementary information was provided by each test (Castaño unpublished data).

Effluents number	*D. magna* LC_{50} 48 h	RTG-2 Cytotoxicity EC_{50} 48 h
1	No Toxicity	Toxicity
2	Toxicity	No Toxicity
41	Toxicity	Toxicity
5	No Toxicity	No Toxicity

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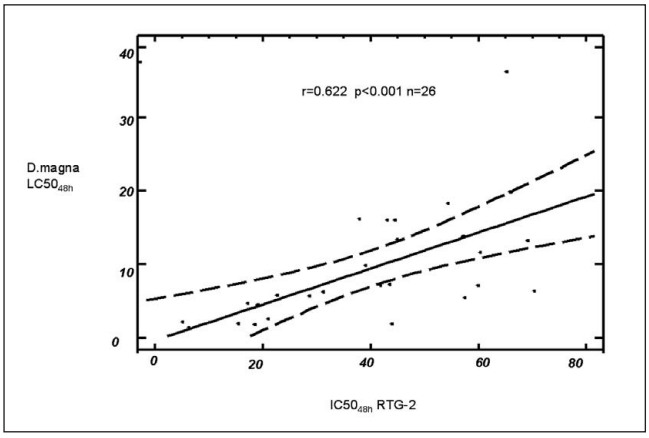

Fig 1: Linear Correlation between EC_{50} from *D. magna* immobilisation test and EC_{50} from Neutral Red Uptake on the RTG-2 fish cell line after testing 26 samples of complex mixtures (industrial effluents), Castaño (unpublished data).

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Ecotoxicological Tests in Non-Ecotoxicological Research: Contribution to the Three Rs

Use of luminescent photobacteria for evaluating the toxicity of 47 MEIC reference chemicals

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Summary
The luminescent bacterium Photobacterium phosphoreum was used for toxicity testing of 47 MEIC reference chemicals. Five-minute EC_{50} values (the concentration of chemical, in mM, which reduces the light output of the bacteria by 50% after contact for 5 minutes) were calculated and correlated with data from the literature: octanol/water partition coefficients, acute $L(E)C_{50}$ data from the Microtox™ and Daphnia tests, acute in vitro toxicity data for fish, animal and human cell lines as well as in vivo data for rodents, dog and man. The log-log correlation coefficients (R^2) ranged between 0.20-0.79. Thus, the application of a photobacteria-based toxicity test (as part of an in vitro test battery) at the screening stage of all areas of in vitro toxicological research (not only in ecotoxicological studies) will contribute to the Three Rs concept.

Keywords: Photobacterium phosphoreum, Microtox™, MEIC chemicals, rodent and human acute lethal toxicity, three Rs

Introduction

Over the last twenty years, alternative, non-animal test systems (mainly eukaryotic cell cultures) have been introduced to supplement and, in some cases, to replace toxicity tests using animals (Carere et al., 2002), contributing to the Three R's concept (replacement, reduction, refinement) introduced by Russel & Burch in 1959.

Several international projects aimed to validate the predictability of *in vitro* tests have been conducted, e.g. the MEIC (**M**ulticenter **E**valuation of ***I**n Vitro* **C**ytotoxicity) programme (1989-1996), initiated by Björn Ekwall and organised by the Scandinavian Society of Cell Toxicology (Bondesson et al., 1989; Ekwall et al., 1989). The MEIC programme was established to evaluate the relevance and reliability of a wide variety of *in vitro* tests for predicting general toxicity in more complex biologic systems. The final goal of the MEIC project was to define optimal batteries of *in vitro* toxicity tests suitable for replacing or supplementing animal tests in predicting the toxicity of chemicals in humans. The rationale behind that approach lay in the fact that toxicity data concerning effects on basic cellular functions and/or structures have a good correlation with *in vivo* data concerning human lethal toxicity (Ekwall, 1999).

Approximately 60 *in vitro* cytotoxicity assays (using human/animal cells and other bioassay endpoints) were tested on 50 compounds of human toxicological interest. MEIC efforts resulted in a series of eight papers in *ATLA* compiling *in vitro* assay, human and rodent toxicity data and comparing their responses (for references, see Ekwall et al., 2000).

In the current work, the reconstituted, freeze-dried (viable) bacteria *Photobacterium phosphoreum* were used for the short-term toxicity testing of 47 chemicals from the MEIC list. Three MEIC reference chemicals were not analysed: dextropropoxyphene chloride (no. 22), thallium sulphate (no. 30) and amphetamine sulphate (no. 47).

P. phosphoreum are naturally luminescent marine bacteria that emit light as a result of their metabolism. Damage to the central cellular metabolism caused by a toxic substance results in a decrease in light output by the bacteria (reduction in light output represents physiological inhibition, not just mortality), the light loss being proportional to the degree of toxicity (Hastings, 1978). In this work the concentration-effect curves for 47 MEIC chemicals were measured and five-minute EC_{50} values (the concentration of chemical which reduces the light output of the bacteria by 50% after contact for 5 minutes) were calculated. These EC_{50} data from the photobacterial test were correlated with data from the literature: octanol/water partition coefficients, $L(E)C_{50}$ data from the Microtox™ and *Daphnia* tests, and acute toxicity data for freshly isolated fish hepatocytes, animal and human cell lines, rodents, dog and man. This work is an update of our previous study (Kahru and Borchardt, 1994) on the toxicity of 39 MEIC chemicals in *P. phosphoreum*.

Materials and methods

Origin of chemicals

MEIC reference chemicals tested are listed in table 1. Chemicals No. 1, 6, 13, 21, 23, 24, 25, 29, 36, 40, 41, 42, 49, 50 were from Sigma; chemicals No. 2, 3, 7, 12, 14, 18, 19, 20, 26, 27, 28, 46 where from Merck; chemical No. 15 (95% purity) was from Kemira (Finland); chemicals No. 5, 17, 31, 34, 37, 38, 43, 44, 48

were from Aldrich; Chemical No. 4 (in the form of pharmacological tablets) was from Rivofarm (Switzerland); chemical No. 32 was from Dr. Schuchardt (Germany); chemical No. 45 was from Boehringer-Mannheim; chemical No. 33 was from Carlo Erba, and chemicals 8, 9 and 10 (HPLC-grade) were from Russia.

Solvents

Chemicals No. 3, 5, 23, 25, 26, 27, 28, 31, 35, 37, 38, 41, 42, 45, 46, 49 were tested in 2% NaCl; chemicals No. 7, 8, 9, 10, 12, 13, 14, 18, 19, 20, 21, 48, 50 were tested in phosphate-buffered 2% NaCl (PBNaCl) (pH=7); chemicals No. 4, 11, 15, 17, 24, 29, 33, 34, 36, 39, 40, 43, 44 were tested in 2% NaCl + 1.5% methanol; chemicals No. 1, 2, 16, 32 were tested in PBNaCl + 1.5% methanol, and chemical Nr. 6 was tested in 2% NaCl + 4% DMSO. The chemicals were handled and the toxicity testing was performed essentially as described in our previous papers (Kahru, 1993; Kahru and Borchardt, 1994).

Test bacteria

Laboratorially prepared freeze-dried photobacterial reagent (*P. phosphoreum* strain FEI 162095 registered in the Finnish Environment Institute) was used. The freeze-dried bacteria were reconstituted by adding 20 ml 2% NaCl into the reagent bottle and incubating it at 4ºC for 20 minutes before use. The average number of bacteria used per assay was about one million.

Statistical analysis

Comparisons between the data sets were made by calculating linear regression using the log-transformed toxicity data. A perfect fit of data where both the photobacterial and other *in vitro* or *in vivo* results were identical would produce an R^2 of 1.0, a slope of 1.0 and a Y-intercept of 0, and the proximity of the calculated values to these absolutes is indicative of the predictive capacity of the X-variable (photobacterial toxicity data) for the Y-variable (data used for the comparison).

Results and discussion

The toxicity of 47 MEIC chemicals was determined using the inhibition of light output of naturally luminescent *Photobacterium phosphoreum* as a toxicity endpoint. The five-minute EC_{50} values, calculated both as mg/L and mM, are presented in table 1. The EC_{50} data for 39 MEIC chemicals obtained in our earlier study (Kahru and Borchardt, 1994) were updated with the toxicity data for paraquat (No. 25), arsenic trioxide (No. 26), warfarin (No. 31), barium nitrate (No. 37), hexachlorophene (No. 38), orphenadrine hydrochloride (No. 42), diphenylhydantoine (No. 44) and sodium oxalate (No. 46) (tab. 1).

Table 1 shows that the five least toxic MEIC chemicals for *P. phosphoreum* (arranged in increasing toxicity, in mM) were potassium chloride (No. 50, EC_{50}=2830 mM), ethylene glycol (No. 7, EC_{50}=2674 mM), methanol (No. 8, EC_{50}=786.5 mM), ethanol (No. 9, EC_{50}=429.8 mM) and sodium chloride (No. 13, EC_{50}=292.6 mM) (tab. 1), whereas the five most toxic MEIC chemicals (arranged in decreasing toxicity, mM) were mercuric

Tab. 1: Five-minute EC_{50} values of the 47 MEIC chemicals determined using the luminescent photobacteria (*Photobacterium phosphoreum*) luminescence inhibition assay

No.	MEIC Chemical	5-minute EC_{50} [mg/L]	5-minute EC_{50} [mM]
1	Paracetamol	2320.00	15.34
2	Acetylsalicylic acid	1140.00	6.33
3	Iron (II) sulphate	2340.00	8.42
4	Diazepam	214.6	0.75
5	Amitriptyline hydrochloride	10.26	0.03
6	Digoxin	1130.00	1.45
7	Ethylene glycol	166000.00	2674.40
8	Methanol	25200.00	786.52
9	Ethanol	19800.00	429.78
10	Isopropanol	11200.00	186.39
11	1,1,1-Trichloroethane	264.70	1.98
12	Phenol	134.00	1.42
13	Sodium chloride	17100.00	292.61
14	Sodium fluoride	9870.00	235.06
15	Malathion	298.73	0.90
16	2,4-Dichloro-phenoxyacetic acid	128.00	0.58
17	Xylene	102.00	0.96
18	Nicotine	434.00	2.68
19	Potassium cyanide	1730.00	26.57
20	Lithium sulphate	30000.00	234.47
21	Theophylline	2150.00	11.93
23	Propranolol hydrochloride	97.54	0.33
24	Phenobarbital	141.19	0.61
25	Paraquat (Methyl viologen)[a]	14830.00	57.66
26	Arsenic trioxide[a]	7.58	0.04
27	Copper (II) sulphate	138.10	0.55
28	Mercury (II) chloride	0.16	0.0006
29	Thioridazine hydrochloride	7.76	0.02
31	Warfarin[a]	90.35	0.29
32	Lindane	60.82	0.21
33	Chloroform	658.10	5.51
34	Carbon tetrachloride	563.30	3.66
35	Isoniazid	4220.00	30.78
36	Dichloromethane	1850.00	21.78
37	Barium nitrate[a]	29400.00	112.49
38	Hexachlorophene[a]	45.89	0.11
39	Pentachlorophenol	10.33	0.04
40	Verapamil hydrochloride	439.30	0.89
41	Chloroquine phosphate	91.60	0.18
42	Orphenadrine hydrochloride[a]	119.40	0.39
43	Quinidine sulphate	88.14	0.12
44	Diphenylhydantoin[a]	21.51	0.09
45	Chloramphenicol	205.00	0.63
46	Sodium oxalate[a]	31500.00	235.07
48	Caffeine	2150.00	11.07
49	Atropine sulphate	3092.00	4.57
50	Potassium chloride	150000.00	28380.00

[a] chemicals that were not analysed for toxicity in our previous paper (Kahru and Borchardt, 1994)

chloride (No. 28, EC_{50}=0.0006 mM), thioridazine hydrochloride (No. 29, EC_{50}=0.02 mM), amitriptyline hydrochloride (No. 5, EC_{50}=0.03 mM), arsenic trioxide (No. 26, EC_{50}=0.04 mM) and pentachlorophenol (No. 39, EC_{50}=0.05 mM).

The *P. phosphoreum* toxicity data for 47 MEIC chemicals were compared with the toxicities of the corresponding chemicals in other test systems (data from the literature) by calculating linear regression using the log-transformed toxicity data. The test systems with respective toxicity endpoints (including the exposure time used for the respective test), the number of data pairs used for the correlation and the equation for the log-log regression line are presented in table 2.

Correlation with lipophilicity (owp)

The octanol water partition (owp) coefficient characterises the lipophilicity of a substance. As a general rule, substances with a higher lipophilicity are more toxic to living cells, mainly due to their enhanced ability to cross the plasma membrane and enter the cell. Table 2 shows that our data are in full agreement with this theory: a relatively high correlation (R^2=0.62) was obtained

Tab. 2: Correlation between EC_{50} values determined using *Photobacterium phosphoreum* (our data) and toxicity data for matching MEIC chemicals determined using other test systems (data from the literature).

Test system	Cytotoxicity endpoint	R^2 log-log	No. of data pairs correlated	Equation for the log-log regression line	Ref.
Physico-chemical properties of a substance					
Physico-chemical properties of a substance	Octanol/water partition coefficient, owp	0.62	27	y = -1.26x + 2.22	Halle et al., 1991
Ecotoxicological tests					
Vibrio fischeri (Microtox™)	Inhibition of light output, 5- or 15-minute EC_{50}	0.64	42	y = 0.86x - 0.07 Fig. 1A	Calleja et al., 1993
Daphnia magna	Immobilisation, 24-h LC_{50}	0.69	38	y = 0.98x - 1.09 Fig. 1B	Lilius et al., 1994
Rainbow trout hepatocytes (freshly isolated)	Plasma membrane integrity, measured by ^{86}Rb-leakage, 3-h EC_{50}	0.53	40	y = 0.79x + 0.63 Fig. 1C	Lilius et al., 1994
***In vitro* assays with animal or human cell lines**					
Rat hepatocytes	Average of three endpoints (morphometric changes, % of viable cells, lactate dehydrogenase release), 24-h CT_{50}	0.66	46	y = 0.73x - 0.34 Fig. 2A	Shrivastava et al., 1992
Chick forebrain primary neuron cultures	Integrity of mitochondria, assayed by tetrazolium MTT, 3-h EC_{50}	0.59	46	y = 0.63x + 0.26	Weiss et al., 1993[a]
Chick forebrain primary neuron cultures	Lysosomal integrity, analysed by neutral red uptake (NRU), 4-h EC_{50}	0.55	47	y = 0.69x + 0.01	Weiss et al., 1993[a]
Various animal or human cell lines	Geometric mean of two or more IC_{50} values per substance. Various endpoints, CT_{50}x	0.79	27	y = 0.83x - 0.22 Fig. 2B	Halle et al., 1991
***In vivo* data**					
Rat	Acute oral LD_{50}	0.37	43	y = 0.45x + 0.10	Weiss et al., 1993[a]
Rat	Acute i.p. LD_{50}	0.29	36	y = 0.38x - 0.14	Weiss et al., 1993[a]
Rat & mice	Acute oral LD_{50}	0.42	47	y = 0.42x + 0.14 Fig. 3A	Shrivastava et al., 1992
Mouse	Acute oral LD_{50}	0.41	42	y = 0.45x - 0.11	Weiss et al., 1993[a]
Mouse	Acute i.p. LD_{50}	0.41	42	y = 0.45x - 0.27	Weiss et al., 1993[a]
Dog	acute lethal i.v. dose, LDL0	0.50	20	y = 0.67x - 0.95	Weiss et al., 1993[a]
Human	Acute lethal plasma concentration, LC	0.32	46	y = 0.52x - 0.96 Fig. 3B	Barile et al., 1994

[a] reported by RTECS (Registry of Toxic Effects of Chemical Substances)

when the toxicity of MEIC chemicals to *P. phosphoreum* was compared to the owp of these chemicals (owp values were taken from Halle et al., 1991).

Correlation with ecotoxicological tests

When the *P. phosphoreum* data were compared with Microtox™ data (from Calleja et al., 1993), the log-log R^2 value was 0.64 (tab. 2; fig. 1A). The high correlation is not surprising, as the test used in the current study and the Microtox™ test are both based on luminescent photobacteria. Moreover, the strain used in the Microtox™ test was formerly also called *Photobacterium phosphoreum* (now *Vibrio fischeri*). We have previously compared the *P. phosphoreum* data (i.e. the strain used by us) with *V. fischeri* (Microtox™) data and revealed a good correlation (log-log correlation coefficient R^2 for 25 laboratory chemicals was 0.94; Kahru, 1993).

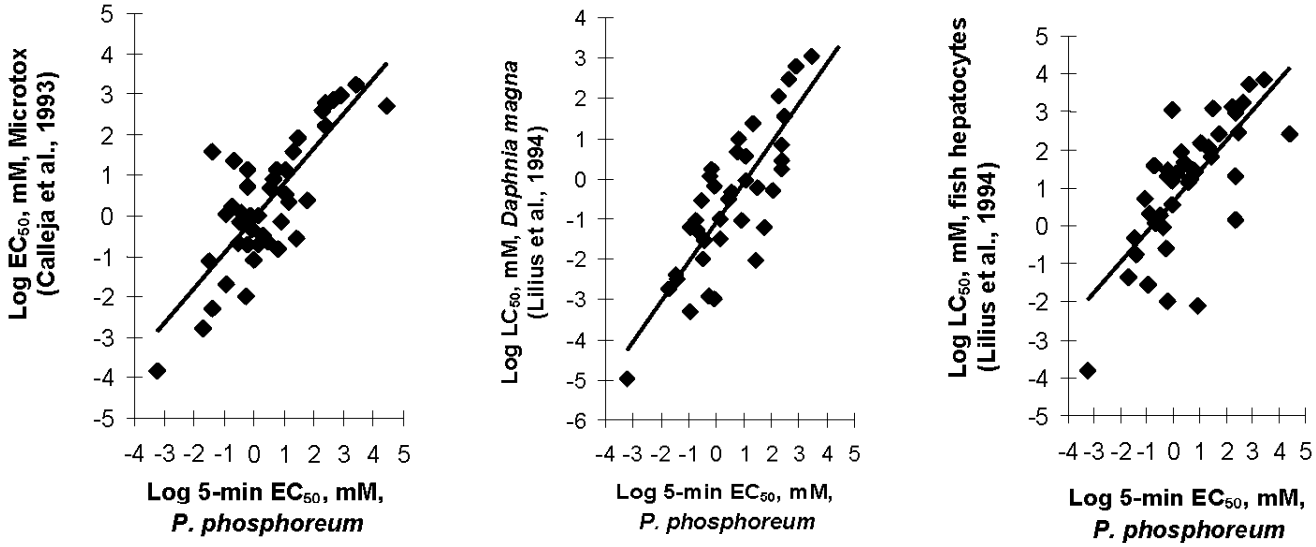
Table 2 and figure 1B show that there was also a relatively high log-log correlation (R^2=0.69) between *P. phosphoreum* toxicity data and that of the *Daphnia* test, a widely used toxicity test in aquatic toxicology, where the immobilisation of aquatic invertebrate *Daphnia magna* after 24 h incubation with chemicals was used as the toxicity endpoint (Lilius et al., 1994). However, the toxicity of most of the chemicals to *Daphnia* exceeded the toxicity to photobacteria by two orders of magnitude. One possible explanation for different sensitivities could be the difference in exposition times (24 hours in the case of *Daphnia versus* 5 min in the case of photobacteria) (tab. 2), i.e. the short exposure to the toxicant may be too short for some types of cellular damages to occur (e.g., caused by bioaccumulation of a toxicant). Correlation of the *P. phosphoreum* toxicity assay with freshly isolated rainbow trout hepatocytes (data taken from Lilius et al., 1994) was reasonably good (log-log R^2=0.53), whereas photobacteria were about 40-fold more sensitive (fig. 1C). It should be mentioned that fish cell lines as well as primary cell cultures are of growing importance in risk assessment of chemicals as substitutes for the acute lethality test in fish usually performed according to OECD guidelines (Fent, 2001; Castaño et al., 2003).

Correlation with cell line assays

Table 2 and figure 2B show that the highest log-log correlation (R^2=0.79) was obtained when *P. phosphoreum* data were compared with the average *in vitro* basal cytoxicity (IC_{50}x, the geometric mean of two or more IC_{50} values per substance, determined by different investigators using various animal or human cell lines and different cytotoxicity endpoints, compiled by Halle et al. (1991). Moreover, the average EC_{50}/IC_{50}x value was 4.3, indicating that the average sensitivity of the photobacterial assay was comparable with that of eukaryotic cell cultures, being at the same time a much less expensive and less laborious toxicity test.

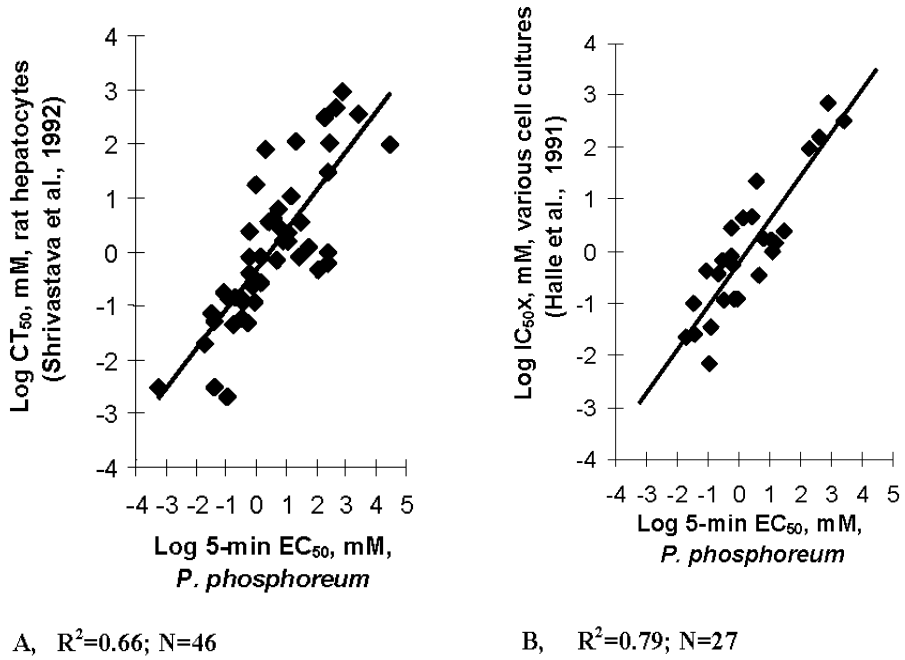
Correlation with *in vivo* data for rodents, dog and human

Table 2 and figure 3 present the comparison of the toxicity of MEIC chemicals in *P. phosphoreum* with *in vivo* toxicity data for the corresponding chemicals obtained from the literature (acute oral LD_{50} and i.p. LD_{50} values for rats and mice, acute i.v. LD values for dog and acute oral and blood plasma LD values for man were used for the correlation, see references in tab. 2). It can be seen that the correlation of five-minute EC_{50} data with the acute oral LD_{50} data for rodents (tab. 2 and fig. 3A) yielded very



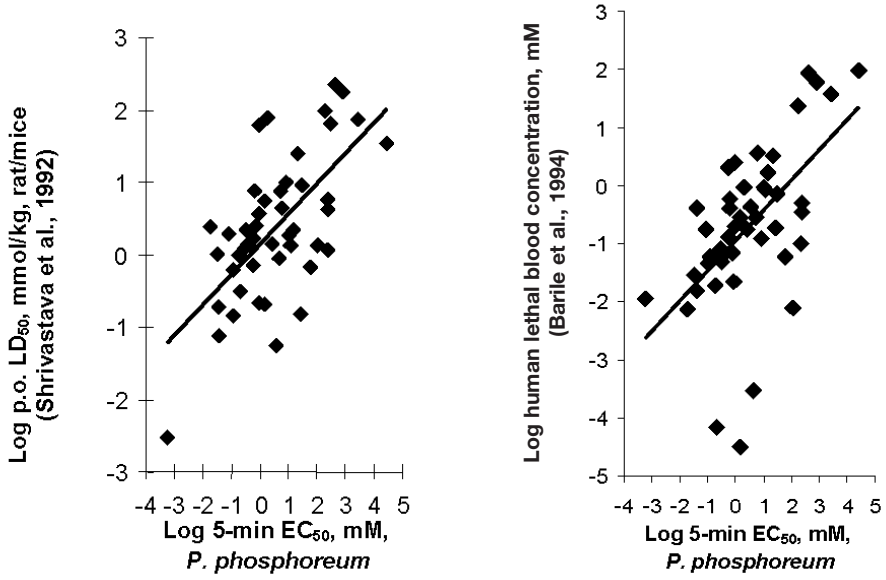
A, R^2=0.64; N=42 B, R^2=0.69; N=38 C, R^2=0.53; N=40

Fig. 1: Logarithms of EC_{50} values (mM) obtained using the *Photobacterium phosphoreum* test *versus* logarithms of $L(E)C_{50}$ values (mM) obtained using the Microtox test for 42 MEIC chemicals (A), *Daphnia magna* test for 38 MEIC chemicals (B) and freshly isolated rainbow trout hepatocyte assay for 40 MEIC chemicals (C). Origin of data used for the comparison and information on the tests are presented in table 2.



A, R^2=0.66; N=46 B, R^2=0.79; N=27

Fig. 2: Logarithms of EC_{50} values (mM) obtained using the *Photobacterium phosphoreum* test *versus* logarithms of CT_{50} values (mM) obtained using rat hepatocyte cultures for 46 MEIC chemicals (A), and logarithms of IC_{50}x values (mM) obtained on various animal or human cell lines for 27 MEIC chemicals (B). Origin of data used for the comparison and information on the tests are presented in table 2.



A, R^2=0.42; N=47 B, R^2=0.32; N=46

Fig. 3: Logarithms of EC_{50} values (mM) obtained using the *Photobacterium phosphoreum* test *versus* logarithms of acute rat/mice p.o. concentrations (mmol/kg) for 47 MEIC chemicals (A) and logarithms of acute lethal blood plasma concentration, LD; mM) in humans for 46 MEIC chemicals (B). Origin of data used for the comparison is presented in table 2.

similar correlation coefficients (R^2= 0.29-0.42) for all rodent data sets, whereas the R^2 values were slightly higher if mouse data were used for the comparison. The highest correlation (R^2=0.50) was obtained, when *P. phosphoreum* data were correlated with dog lethal i.v. values (referenced in Weiss and Sayer, 1993) (tab. 2).

The correlation of photobacterial data with human data (tab. 2 and fig. 3B) was not impressive (R^2= 0.20-0.32). However, the predictive potential of the chick forebrain primary neuron culture MTT (see also tab. 2) EC_{50} data for the determination of toxic doses in humans (R^2=0.29; Weiss and Sayer, 1993) was comparable to that of *P. phosphoreum*. Also, the predictive

potential of rat i.p. LD_{50} data for man oral LD (R^2=0.19; Weiss and Sayer, 1993) was comparable to that of *P. phosphoreum*. It must also be stressed, that, due to ethical necessity, estimations of human chemical toxicity are based on ill-defined populations of people and, often, only estimates of dosage. This could be one of the reasons for the relatively poor correlation observed.

Conclusions

This paper is an update of our previous study in which the toxicity of 39 MEIC chemicals was studied using naturally luminescent marine bacteria, *Photobacterium phosphoreum*, as test organisms (Kahru and Borchardt, 1994). In the current study, that data set was updated with the toxicity data for an additional 8 MEIC chemicals (paraquat, arsenic trioxide, warfarin, barium nitrate, hexachlorophene, orphenadrine hydrochloride, diphenylhydantoine and sodium oxalate) (tab. 2) and correlated with data from the literature: octanol/water partition coefficients, $L(E)C_{50}$ data from the Microtox™ test and *Daphnia*, and acute toxicity data for freshly isolated fish hepatocytes, animal and human cell lines, rodents, dog and man.

Our data showed that luminescent bacteria (e.g., *P. phosphoreum*, *Vibrio fischeri*) could be used at the screening level to test the safety of chemicals, as this assay system is very cheap, easy to use, well reproducible, very rapid, and could be more easily automated than conventional testing systems (e.g. cell cultures, experimental animals). These photobacterial tests also yield very similar results (this paper, Kahru, 1993; Kahru et al., 1996 and Jennings et al., 2001). Moreover, the *V. fischeri* NRRL B-111 77 test is commercially available under several trade marks (ToxAlert™, Microtox™, LUMIStox™, BioTox™), and photobacterial toxicity data exist for more than 1000 chemicals (Kaiser and Devillers, 1994). Therefore, the application of photobacteria-based toxicity tests (as part of an *in vitro* test battery) to all areas of *in vitro* toxicological research, not only ecotoxicological studies (Bispo et al., 1999; Kahru et al., 2000; Castillo et al., 2001; Loibner et al., 2004), should be seriously considered, as this could save a lot of money, manpower and lives of experimental animals and thus contribute to the Three R's concept (replacement, reduction, refinement) introduced by Russel & Burch in 1959.

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Application of *In Vitro* Alternative Methods to Ecotoxicology

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Summary

We applied in vitro assays using cultured cells for the ecotoxicological assessment of various samples collected from the environment. The results of cytotoxicity assays on water samples from rivers and cooling towers correlated well with the respective water analyses. However, some samples induced different responses between the two cell lines employed. Extracts of solid samples revealed the order of contaminations in the phototoxicity test and mouse lymphoma mutation assay. Therefore, the in vitro toxicological assays were useful to screen test samples from the environment composed of various pollutants.

Keywords: cytotoxicity, phototoxicity, environmental pollutants, ecotoxicology

Introduction

Detection of environmental pollutants is very important in ecotoxicology. Environmental samples may be in the form of gas, liquid, sludge, particle and so on (Al-Khodairy and Hannan, 1997; Monarca et al., 1998; Minissi et al., 1998; Vahl et al., 1997), and this makes it difficult to examine the samples and to compare results.

Until now, mainly chemical analyses have been used to assess pollutants in water and air. Although chemical analyses are becoming more accurate due to advanced analytical technology, they detect only the targeted chemicals in the test sample, which may contain a combination of various chemicals.

Fish, birds, invertebrate animals, and plants, etc., have been used to assess the toxicity of chemicals in the environment (Huang et al.,1996; Ueda et al., 2005; Ralph and Petras, 1997; Yoshimura and Endoh, 2005; Wang et al., 2005). However, these kinds of tests aimed at assessing one target chemical in the environment and not to assess the complex mixture that exists in the environment. In view of the 3Rs, animal testing should not be conducted, if at all, without obtaining chemical information and results of *in vitro* testing first.

Meanwhile, many *in vitro* methods have been used as prescreening methods before animal testing or as alternative testing methods. We applied mammalian cell culture systems using several *in vitro* methods to detect toxicity with high sensitivity and compared the results with the chemical analyses of the samples. Since it was difficult to use all sample forms in one or a few methods, we needed to adjust the sample forms to testing method, e.g. to extract with solvent.

Two types of samples, liquid and solid, were collected from the environment. Liquid samples were used after filtration and extracts were made of the solid samples. Chemical analysis was performed on all samples as well.

Materials and methods

Liquid samples

Water samples from cooling towers on buildings and river water samples were collected. A cooling tower is a system often set on top of buildings to cool down water that has been warmed when circulating in the building. There are two types of systems, open systems and closed systems. In open systems, the warm water runs downward while air is blown through the water to cool it. It is not covered firmly, since the water is cooled down with outside air, so tiny droplets of water can spread to the environment. We collected the water of cooling towers from six facilities, four located in Tokyo, Japan and two in Kanagawa prefecture near Tokyo.

River water samples were collected from Sakawa river and its tributaries in Kanagawa prefecture (Wakuri et al., 2002). Thirteen points were chosen and water samples were collected six times over two years. At one assessment time, samples were collected from only nine points.

Colony formation test and chemical analysis were performed with these samples. The liquid samples were sterilised by filtration before use in the *in vitro* test.

Solid samples

Three types of samples, river sediments, ashes from incineration facilities, and airborne particulates were collected.

River sediments were collected in the same rivers from which the water samples were collected. We chose four points out of the points used for water collection, so that we could compare the results with those of the water analysis. The sediments were dried and extracted with organic solvent. The extracts were tested in colony formation and phototoxicity tests.

Ashes were collected from six incineration facilities. Two types of ashes, bottom ashes and fly ashes, which were collected

with an electrostatic precipitator, were obtained from four facilities. From the other two facilities, we collected bottom ashes or fly ashes. They were extracted with organic solvent and the extracts were employed in the phototoxicity test.

Airborne particulates were collected in Sapporo city, Hokkaido, which is the northernmost prefecture in Japan (Matsumoto et al., 1998). The airborne particulates that had been collected over 24 years using a high-volume air sampler and filters had been stored at -20˚C. They were extracted with organic solvent and each test sample was made up with 2 years' worth of extract. Phototoxicity test and mouse lymphoma assay were performed with these extracts.

Colony formation test

The colony formation test was used as a cytotoxicity test. BALB/3T3 A31-1-1 (BALB/3T3) cells derived from mouse embryo and VERO cells derived from the kidney of an African green monkey were used in the colony formation assay. Both cell lines, especially BALB/3T3 cells, are known to be sensitive to chemicals.

One day prior to treatment, 100 cells were seeded onto a 60-mm dish. The cells were treated with the extracts or the medium prepared with filter-sterilised water samples. The cells were treated for 7 days in case of BALB/3T3 cells and for 10 days in case of VERO cells. After incubation, cells were fixed, stained and colonies were counted.

Phototoxicity test

The *In Vitro* 3T3 NRU phototoxicity test (3T3 NRU PT), which was adopted as an OECD guideline in 2004, was used to detect phototoxicity. BALB/3T3 cells were plated into 96-well plates and the next day cells were treated with extracts.

For treatment, the medium was changed to test solution, then cells were pretreated in an incubator for 1 h. After pretreatment, one plate was irradiated at 1.7 mW UVA/cm^2 for 50 min, and another plate was kept in the dark for 50 min to assess cytotoxicity without UV irradiation.

After treatment, the cells were incubated for one more day and cell survival rates were measured by the Neutral Red uptake method.

Mouse lymphoma assay

We used a mutation assay based on L5178Y/tk$^{+/-}$-3.7.2C mouse lymphoma cells with trifluorothymidine resistance known as the mouse lymphoma assay (MLA).

The extracts were treated with or without S9 mix for three hours, then washed out. The cells were cultured for two days and then were replated to detect mutation induction.

Chemical analysis of water

A series of water analysis was performed under the Japanese water quality guideline.

We used three parameters, chemical oxygen demand (COD), iron and lead, for water from cooling towers and four parameters, number of bacteria, COD, nitrate and nitrite nitrogens, and electric conductivity for river water.

Chemical analysis of extract

The amount of polycyclic aromatic hydrocarbons (PAHs) was measured in the extracts from ashes and airborne particulates. Seven PAHs (phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, benz(a)pyrene, acenaphthylene) were analysed in the ashes and eight PAHs (benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene chrysene, fluoranthene, pyrene) in the airborne particulates.

Results

Water from cooling towers

The IC_{50} values in the colony formation tests with BALB/3T3 and VERO cells were comparable in the water samples of four of the six buildings. The other two samples showed no toxicity in one cell line and strong toxicity in the other. This suggested that the two cell lines display differing sensitivities to some substances.

The results of the chemical analysis of lead, iron and COD showed tendencies similar to the colony formation tests. The samples that showed higher contents of lead, iron or COD, which means that the waters were "dirty", also showed stronger cytotoxicity.

Water from rivers

After obtaining all results, the water samples were ranked. First the ranking was made for each time point for each parameter. The ranking numbers of two consecutive years were summed-up for each point, then they were renumbered according to their sum. Ranks were also determined for overall cytotoxicity and for overall chemical content.

Though the regression coefficient between the results for BALB/3T3 and VERO cells was less than 0.6, and the correlation between the cytotoxicity ranking and each single chemical parameter was about 0.5-0.8, the total ranking of cytotoxicity and the overall chemical content showed a good correlation with a regression coefficient of 0.9.

Sediments from rivers

VERO cells showed higher sensitivity (2-5 times as IC_{50} values) than BALB/3T3 cells for all four sample points. However, the sediments collected at upper stream sample points showed less cytotoxicity than those collected at lower points in the stream in both cell lines. This tendency was also reflected in the results of the chemical analysis of the water collected at the same points.

Meanwhile, the results of the phototoxicity assay were similar to those of the cytotoxicity test in the upper three points. The lowest point showed less phototoxicity than the middle two points. This might be because the sediment material was different from the middle two points. The sediments at the middle two points were mud-like but the lowest point included more sand-like particles. This implies that the absorption conditions could have been different and, therefore, the results did not reflect the water analysis.

Ashes from incineration facilities

Ten ash samples from the incineration facilities were examined. Fly ashes contained more PAHs than bottom ashes except for those of one facility. In some facilities, the extracts showed stronger phototoxicity, as ashes contained large amount of PAHs. However, the extract that contained the highest amount of PAHs was the least cytotoxic under irradiation conditions and almost the least cytotoxic under non-irradiation conditions.

Airborne particulates

In the phototoxicity test, cytotoxicity without irradiation was stable in the samples collected over 24 years. On the other hand, cytotoxicity under irradiation decreased over the 24 years in the same manner as the amount of PAHs decreased.

Mutation induction in the presence of S9 mix showed the same downward trend as the amount of PAHs and cytotoxicity under irradiation. There were no clear tendencies regarding mutation induction in the absence of S9 mix, though the old samples (first 6-8 years) showed higher mutation induction.

Discussion

The cytotoxicity of liquid samples from cooling towers and rivers displayed the same tendency as the chemical analysis. The two cell lines used did not show a high correlation, but the difference in their sensitivity can be thought of as a differing ability to detect some pollutants. Since the chemical analysis of the water has been used to judge the water quality, *in vitro* cytotoxicity testing could be used to detect water pollution, if the cell line is chosen carefully.

Regarding the solid samples, the cytotoxicity of river sediments corresponded with the water analysis, though phototoxic effects showed some discrepancies. One reason might be that we did not analyse the extracts themselves and phototoxicants could be accumulated in sediments. Another is that the water analysis did not include detection of contaminants that are phototoxic.

Phototoxicity results of ashes did not agree well with the results of the chemical analyses either. Though PAHs are known as strong phototoxicants (Yu, 2002; Nikolaou et al., 1984), the PAHs we analysed might not be the major contributors to the phototoxicity or the major substances causing phototoxicity might not have been analysed. Since the conditions of incineration and the rubbish burnt were different in each facility, the contents of the ashes could be different as well.

Airborne particulates were tested by phototoxicity and mutation assay. Both results suggested that PAHs contributed part of the activities. PAHs are well-known to show photo-enhanced cytotoxicity and mutation reaction with S9 mix (Penning et al., 1999). In our results, cytotoxicity under irradiation conditions, mutation induction in the presence of S9 mix and the amount of PAHs displayed the same tendencies. This suggests that PAHs were the main cause of phototoxicity and mutation induction in airborne particulates.

Conclusion

Firstly, the results of the *in vitro* testing generally agreed with the results of the water/chemical analysis. Differences might have been caused if the substances analysed chemically did not induce toxic effects *in vitro*.

Secondly, *in vitro* tests can be used to detect various biological reactions by testing a complex sample mixture with several test methods. The chemical analysis shows only the quantity of target substances and not the effects of the substances, whereas the *in vitro* tests show biological reactions that we could use to predict the *in vivo* reaction.

Finally, we expect that *in vitro* testing would make it easier to compare the results from different environmental samples without requiring any animal testing.

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