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Frozen mitochondria as rapid water quality bioassay

Alessandra Iero, Sabrina Manente *, Guido Perin, Marcantonio Bragadin

Department of Environmental Sciences, Dorsoduro 2137, Venezia 30123, Italy

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Abstract

A rapid and relatively low cost bioassay, usable in routine screening water test has been developed modifying the beef heart mitochondria test. In our experiments, mitochondria (FM22) were frozen at −22 °C, instead of −80 °C (FM80), and their applicability and sensitivity was verified. The oxygen consumption was measured by a Clark electrode that was interfaced to a PC to collect test analysis data. Blank tests were carried out to verify the oxygen consumption linear fitting. Toxicity tests were performed using pure organic and inorganic compounds, such to verify the FM22 sensitivity. A piecewise regression, through an Excel® Macro, identified the break-point in the oxygen consumption and calculated the toxicity. The IC_{50}s of the tested compounds were calculated and ranged from 0.123 to 0.173 mg/l for heavy metals (Cd, Cr, Cu, Ni, Pb and Zn) and from 0.572 to 10.545 mg/l for organics (benzene, DMSO, DDE, endrin, dichloromethane, chlorobenzene, 1,2-dichlorobenzene and 1,3-dichlorobenzene). Water effluent samples were then tested. The FM22 gave different toxic reactions to them. Water samples were characterised for heavy metals. The FM22 bioassay had a higher sensitivity than the FM80 and a high reproducibility in the toxicity test with pure compounds. The FM22 test was a good predictor of toxicity for water samples; the bioassay is easy, low cost and rapid, then usable for routine tests.

Keywords: Toxicity test; Validation; Rapid screening; Effluents

1. Introduction

Organic and inorganic contaminants found in water result from domestic, industrial and agricultural wastes, accidental spillage or superficial runoff. Many of these substances eventually end up in the sediments and in surface and subsurface reservoirs, due to an increase of organic and inorganic contaminants in the biosphere, leading the problem of assessing the toxicity induced by these compounds.

Several methods have been proposed, using responses of whole organisms such as algae, fish and invertebrates, as extensively described in Cooney (1995) for freshwater systems. The problem, however, is not only strictly scientific, but also involves cost, resources and time. For example, fish require expensive testing facilities and long operational times are necessary for toxicity measurements.

Thus, more rapid and less expensive biosensors have been proposed (Bulich, 1979; Blondin et al., 1985, 1987; Yamano and Morita, 1993; Argese et al., 1995). Some biosensors use the responses of whole cells and of mitochondria (Knobeloch et al., 1990; Bragadin et al., 1991; Betterman et al., 1996; Riisberg et al., 1996; Palmeira and Madeira, 1997; Wenzel et al., 1997; Manente et al., 1999).

Mitochondria play a central bioenergetic role in the organisms. Since they produce ATP for the cells, any damage to their activity induced by toxic compounds may affect the entire organism (Chance and Williams,

* Corresponding author. Tel.: +39-041-2348634; fax: +39-041-2578649.

E-mail address: manente@unive.it (S. Manente).

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1955; Slater, 1967; Van Dam and Wiechmann, 1979; Mitchell, 1979; Hatefi, 1993). Therefore, mitochondria are good predictors for acute toxicity in solution as shown by Moreno and Madeira (1991), Shannon et al. (1991), Jeevaratnam and Vidya (1994), Bragadin et al. (1998), da Silva et al. (1998) and Bragadin et al. (1999a,b).

The method (FM80), using the response of −80 °C frozen beef heart mitochondria has been proposed by one of the co-authors of this paper (Bragadin and Dell'Antone, 1994). Frozen mitochondria cannot produce ATP anymore, but the rate of ATP synthesis could be correlated by a stoichiometric ratio to the respiratory rate (that will be called "oxygen consumption" henceforth). The frozen mitochondria test, will eventually measure the inhibition in the respiratory rate induced by toxic compounds.

The goal of our research was to validate a test, similar to the FM80, having −22 °C as frozen temperature for mitochondria. The test will be referred to as FM22 henceforth. This test will cut not only material costs, as mitochondria from the same preparation can be used for several months, but also, considerably, storage costs, since the −22 °C is a less expensive freezer. Thus, considerations regarding the costs of storage and the rest of the laboratory equipment, and the time needed to carry out the experiments, led to consider this test suitable for fast screening and monitoring.

A series of experiments have been carried out using pure organic and inorganic compounds, in order to verify reproducibility and sensitivity of the method. Data were treated to calculate IC_{50} (inhibition concentration) of the compounds. Then, different water samples, mainly from industrial wastewater, were tested. The samples were characterised for metal contents by AAS trying to get, through chemical analysis, information on the potential risk.

2. Material and methods

Sucrose, tris-HCl and Na-succinate for molecular biology were purchased from BDH. Pure reagents for atomic absorption were purchased from Sigma–Aldrich. Milliq water was used.

The mitochondria were prepared accordingly to Azzone et al. (1979) from bovine heart despite the classical hepatic source, due to two reasons: (a) possibility to obtain a higher quantity of mitochondrial proteins during a single procedure and (b) heart muscle mitochondria are considerably more resistant than liver ones to aging. Bovine heart mitochondria preparation steps can be resumed as follows. The bovine heart, once removed, is immediately placed in ice. It is essential to maintain the heart at 2–4 °C through all steps. Once in the laboratory, the heart is carefully cleaned from fat and connective tissues and cut into little cubes. The cubes are then passed through a meat grinder (4–5 mm plate holes, 250 rpm) and the minced meat is weighed (in order to reach pools of ≈500 g). Each pool is then put in 0.25 M sucrose, 5 mM tris-HCl, 2 mM EDTA-tris, pH 7.5 and shaken for 10 min. The pH is eventually readjusted with 5 M triethanolamine. Each pool, after being squeezed through a cheesecloth and freed from washing solution, was rearranged in 200 g pools, to which 500 ml of solution A (0.25 M sucrose, 10 mM tris-HCl, 1 mM succinate-tris, 2 mM EDTA-tris, pH 7.5) is added and blended at 8000 rpm for about 45 s, pH readjusted to 7.5 with 5 M triethanolamine. The homogenate is centrifugated (1300 rpm for 20 min) and the supernatant solution is removed and filtered (pH eventually readjusted to 7.5 with 5 M triethanolamine) in order to obtain a suspension. The suspension is then centrifugated (15 000 rpm for 15 min) and the obtained supernatant is removed, while five times the volume of the pellet is resuspended adding solution A. This mitochondrial suspension is homogenized in a glass–teflon homogenizer (200 rpm) and then centrifugated again (15 000 rpm for 15 min). The final pellets are then resuspended in 0.75 M sucrose, 10 mM tris-HCl, pH 7.5. Mitochondrial protein concentration was determined by the Lowry method (Lowry et al., 1951). Once prepared, the mitochondria were placed in 2.5 ml Eppendorf vials and frozen at −22 °C in our specific procedure.

The YSI 5331 Clark oxygen electrode was used. Blue Ribbon filter paper (retention 16 μm) was obtained by Schleicher & Schuell GmbH. Heavy metals were determined by a Varian Spectr AA-250 Plus, bringing pH of samples to 2.0 by HNO_3.

A scheme of the Pyrex vessel used for the toxicity test is shown in Fig. 1. The 2.5 ml Pyrex vessel, closed by a teflon® cap, was thermostated at 25 °C; the solution was magnetically stirred. A very small hole in the vessel's cap allowed to insert the syringe needle for adding mitochondria, succinate and the testing solution.

2.1. Bioassay methodology

Mitochondria contained in the Eppendorf vials were quickly thawed out using 70 °C hot water for 30 s and then maintaining them at 0 °C in an ice–water bath while running the tests. For the test, thawed mitochondria were mixed by vortex and an aliquot, able to yield to a 0.1 mg of mitochondrial protein in the 2.5 ml of the vessel, was syringed and resuspended in 0.25 M sucrose and 10 mM tris-HCl, at pH 7.4. No ATP was added; the bioassay was carried out in the state 4 of respiration (Lehninger, 1979), activated by adding succinate 1 mM.

Mitochondrial response to toxic substances was expressed as variation in the rate of oxygen consumption.

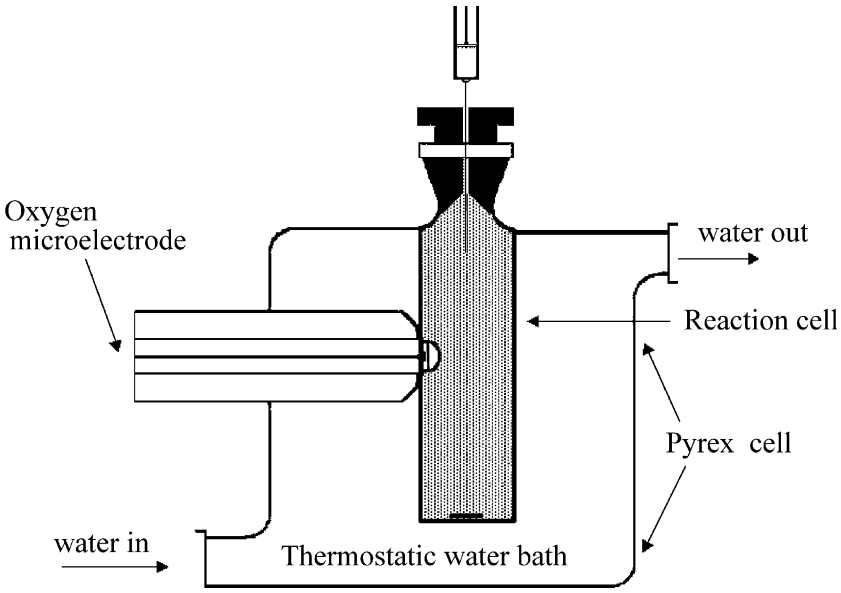

Fig. 1. The toxicity test vessel.

A Clark electrode polarographically monitored oxygen consumption (Lyons et al., 1974). It was interfaced to a PC by a PC multilab card "pcl-711s", that allowed to follow the oxygen trend in real time and to record the numerical data of each test. Each test was carried out for 20 min, sampling one datum every second, thus getting 1200 numerical data for each test. The electrode output signal was amplified and conducted to the computer through the card. The multilab card is a low cost, multifunction PC plug-in I/O card turning the PC into a data acquisition system.

2.2. Bioassay procedure

A calibration step performing blank tests and tests on pure organic and inorganic compounds was carried out, in order to verify the FM22 reproducibility and their sensitivity.

During blank tests, only the medium, the mitochondria and the succinate were placed in the reaction vessel, to verify the linear fitting of the oxygen consumption rate. Toxicity tests started like a blank, with medium, succinate and mitochondria; after 5 min the toxicant to be tested was added in the vessel. An inhibition in the oxygen consumption induced by toxic compounds will eventually be expressed by a change in the slope of the oxygen trend. Therefore in the toxicity tests, the first part of the oxygen consumption trend represented the blank and was then compared to the second part, expressing the action of the toxicant. Pure compound toxicities were quantified comparing the slope before and after adding the compound. For each tested concentration, at least six replicates were run. Table 1 shows the concentration range used for each organic and inorganic tested pure compounds, the total number of replicates, the IC_{50} (expressed in μg/l) calculated by linear regression, and the standard deviations.

As numerical data of each test have been gathered, the toxicity was quantified by a statistical methodology that automatically identifies a "break-point" in the linear regression and quantifies the variation. The IC_{50} were calculated on the tested compounds (inhibition concentration 50%, the concentration of the compound reducing the oxygen consumption by 50%, as compared to its blank). Fig. 2 shows a typical inhibition trend. Y-axis has arbitrary units.

Once the bioassay sensitivity was verified using heavy metals and organic compounds, water samples from various effluents were tested. The water effluents were first filtered with Blue Ribbon filter paper and then 20 l of sample was tested following the same methodology of pure compounds.

2.3. Statistical methodology

The linear fitting of O_2 consumption rate was verified for −22 °C frozen mitochondria: blank bioassay data were linearly regressed and the R^2 was used as indicator. Afterwards, toxicity tests were run. In each test the change in the oxygen consumption slope, due to toxicant action, was quantified by a "piecewise regression" (Quandt, 1958; Seber, 1989). An Excel® macro was set up and applied the regression to the data-set. This allowed to identify the break-point, expression of toxicant action, and to quantify the slope variation. The "piecewise regression" finds the break-point (Beckman and

Table 1
Each compound was screened within a wide range of concentration; each concentration was tested at least in six replicates

Compound	Tested concentration range	Total number of replicates	IC_{50} (ppb)	Standard deviation
Zn^{2+}	0.24 < conc (μg/l) < 0.28	18	0.173	0.080
Cr^{6+}	0.20 < conc (μg/l) < 0.32	24	0.123	0.087
Ni^{2+}	0.20 < conc (μg/l) < 0.32	24	0.125	0.056
Cd^{2+}	0.20 < conc (μg/l) < 0.40	24	0.153	0.029
Pb^{2+}	0.2 < conc (μg/l) < 0.6	24	0.143	0.080
Cu^{2+}	0.008 < conc (μg/l) < 0.40	48	0.128	0.152
Benzene	0.35 < conc (μg/l) < 7.02	42	0.572	0.192
DMSO	5.80 < conc (μg/l) < 11.60	24	4.515	0.221
DDE	18.00 < conc (μg/l) < 90.10	24	13.457	0.302
Endrin	7.00 < conc (μg/l) < 10.00	24	10.545	0.263
Dichloromethane	1.00 < conc (μg/l) < 3.20	24	4.211	0.376
Chlorobenzene	0.66 < conc (μg/l) < 3.55	36	0.743	0.077
1,2-Dichlorobenzene	0.52 < conc (μg/l) < 1.41	24	0.686	0.054
1,3-Dichlorobenzene	0.510 < conc (μg/l) < 1.150	24	0.718	0.071

A linear regression was applied to calculate IC_{50}. More detailed explanation in the test.

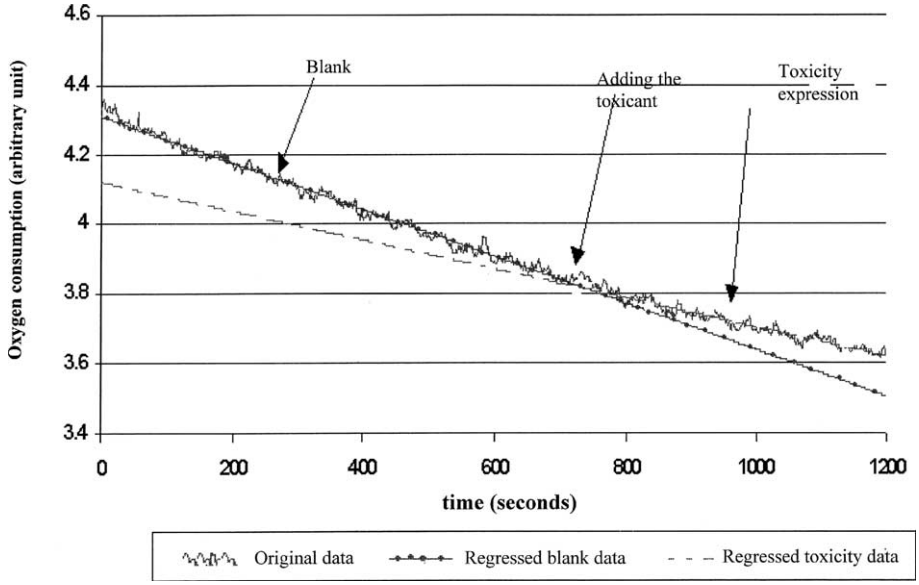

Fig. 2. A FM22 toxicity test output. The figure represents a typical direct output of the test (original data), and the data calculated through a linear regression after the piecewise regression identified the break-point (regressed blank data and regressed toxicity data). *Y*-axis is in arbitrary units.

Cook, 1979; Worsley, 1983) using a hypothesis test based on a *F* statistic. Because the linear fitting had been verified for the bioassay, it was possible to apply this statistical procedure. Validation of the procedure was made through the large amount of numerical data gathered for each test (1200).

2.4. Sampling methodology

Surface water, industrial and municipal wastewaters were tested to verify the validity of the test method when applied to environmental samples. Diverse effluents were sampled, to evaluate the possibility of using the bioassay as a screening test for possible source of risk for the environment. Thirteen samples were taken in the vicinity of Venice (Italy) area and included three dumping sites (one municipal, sampled before and after the treatment, another municipal and one of special residues), three sewage treatments (one municipal, one industrial, one municipal and industrial, all sampled before and after the treatment) and three electroplating factories. Further 12 samples were taken in the Rovereto (Trento, Italy) area and included two surface water samples, one electroplating factory effluent, and nine different effluents

from industrial plants (two food treatment, one pharmaceutical factory, one pulp mill and paper manufacture, two mechanical, two textile, one cleansing plants). Each sample was obtained on four hours sampling, to representatively average the effluent. Samplings were carried out following the standard methods for water and wastewater (Eaton et al., 1995).

3. Results

3.1. Blank tests

One hundred blank tests were performed and the R^2 was used as indicator of linearity. A descriptive statistic (Table 2) was obtained for the R^2 values that verified the blank linear fitting. The mean value of R^2 is very high and has a low standard deviation.

The preliminary assumption of a linear fitting for the oxygen consumption was confirmed from the results and it was possible to follow through with toxicity assays.

3.2. Test calibration with pure compounds

Toxicity tests have been run using pure heavy metals and organic compounds, usually found in environmental samples.

Table 2
Descriptive statistics for R^2 on blanks ($n = 100$)

Mean	0.9893
Standard deviation	0.0095
Variance	9.15E−05
Coefficient of variance	0.9668

Zinc (as Zn^{2+}), chromium (as Cr^{6+}), nickel (as Ni^{2+}), cadmium (as Cd^{2+}), lead (as Pb^{2+}), copper (as Cu^{2+}) have been tested in their ionic form, as they are well known for interfering with the normal functioning of mitochondria and cells. To prepare solutions we have used metal nitrate salts; indeed, it is known nitrates do not interfere with mitochondria respiration (Carpenedo and Floreani, 1989; Lodish et al., 1996) and the same kind of test performed with metal plus EDTA confirm this fact.

The non-chlorinated compounds benzene (polarity index = 0.3) and dimetilsulfoxide (DMSO) (polarity index = 6.5), the chlorinated solvents, dichloromethane, chlorobenzene, 1,2-dichlorobenzene, 1,3-dichlorobenzene, and two pesticides, DDE and endrin, have been tested.

All of the organic compounds are lipophilic, which may affect bioenergetic mitochondria functions, by alteration of the hydrophylic/hydrophobic interaction of the involved enzymes (Stolze and Nohl, 1994).

The IC_{50}s (in μg/l) calculation is performed (see Table 1) by linear regression among all tested concentrations (each one with at least six replicates). Standard deviation values are reported.

3.3. Field water samples

Toxicity results of the samples obtained from the two different industrialised zones are given in Fig. 3 and their legend in Table 3. Because they are complex environmental samples, their action on the oxygen consumption is indicated as inhibition percentage (%) and scaled between 0 and 100.

Metal content (Table 4) analysis shows high concentration of Ni^{2+}, Zn^{2+} and Cr^{6+}, as expected, in

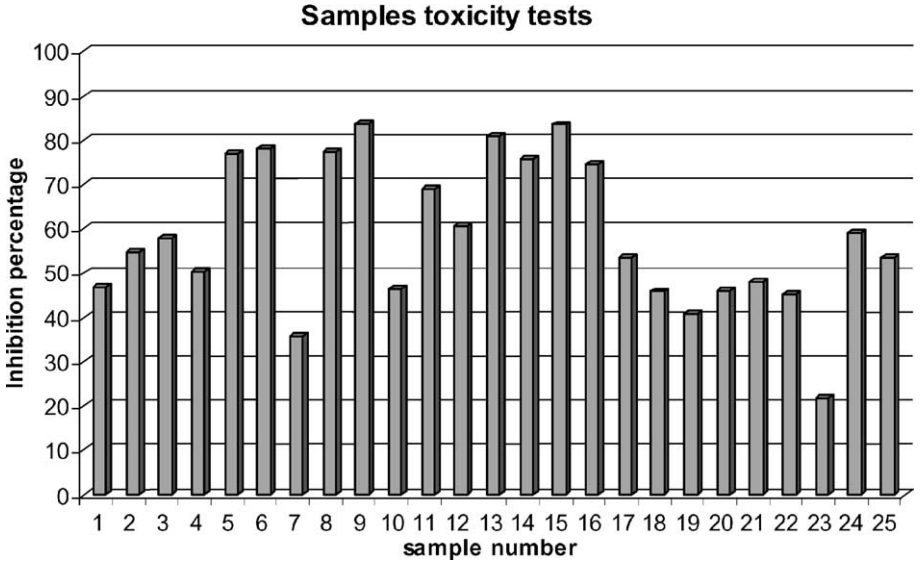

Fig. 3. Toxicity on FM22 bioassay of water samples. Results on inhibition percentage (%).

Table 3
Sources of samples

Location number	Description
1	Municipal dumping site (COVENOR, Ve) lecheate, treatment influent
2	Municipal dumping site (COVENOR, Ve) treatment effluent
3	Domestic sewage treatment (Caorle, Ve), influent to treatment
4	Domestic sewage treatment (Caorle, Ve), effluent from treatment
5	Dumping site (Ca' Rossa, Ve), lecheate
6	Electroplating 1, influent to treatment
7	Electroplating 1, effluent from treatment
8	Electroplating 2, effluent from treatment
9	Special residues dumpings (ECOVENETA, Ve), lecheate
10	Industrial sewage (ENICHEM, Ve), influent to treatment
11	Industrial sewage (ENICHEM, Ve), effluent from treatment
12	Industrial/municipal sewage (Fusina, Ve), influent to treatment
13	Industrial/municipal (Fusina, Ve), effluent to treatment
14	Costa creek, Rovereto Tn, upstream of small industrial area
15	Costa creek, Rovereto Tn, downstream of small industrial area
16	Textile factory (GETECA, Rovereto Tn) effluent
17	Food treatment factory (ICARCONSERVE, Rovereto Tn), effluent
18	Pharmaceutical factory (ROFERM, Rovereto Tn), effluent
19	Dye factory (AQUASPACE, Rovereto Tn), effluent
20	Mechanical activity (Microleghe Trentine, Rovereto Tn), effluent
21	Mechanical activity (Meccanoptica, Rovereto Tn), effluent
22	Cleaning activity (Pulisprint, Rovereto Tn), effluent
23	Food treatment factory (Funghi Trentini, Rovereto Tn), effluent
24	Electroplating (Galvanica Trentina, Rovereto Tn), effluent
25	Pulp mill and paper manufacture (Aticarta, Rovereto Tn), effluent

Samples 1–13 are from an area in the vicinity of Venice; samples 14–25 are from an area in the vicinity of Rovereto (Trento).

samples 6, 7, 8 collected in the vicinity of Venice from electroplating factories. However, in all samples heavy metals were detected, except for cadmium that was almost always below detection limit. In the Rovereto area samples (number 14–25), metals were mostly below detection limit, except for zinc that ranged between 3 and 86 μg/l.

4. Discussion

The possibility of using the FM22 bioassay has been widely explored. Since no information on the activity of mitochondria frozen at −22 °C was available, blank tests were run first. The very high values of R^2 confirmed the oxygen consumption linear fitting. Afterwards, toxicity tests of pure compounds, based on the reduction of the respiratory rate, which had been quantified considering the slope change, were then run to check the method sensitivity.

A −22 °C frozen bovine heart mitochondria batch can be stored before using it up to six months since it's preparation (data extrapolated from six preparations). Mitochondria from the same preparation and mitochondria from different preparations have shown variability in the oxygen consumption rate ranging between 5% and 15%. However this variability does not represent an issue of concern as far as the slope variation, for toxicity calculations, is checked within each test run and then each mitochondria mini-pool works intrinsically.

Results from pure organic and inorganic compounds toxicity tests show a high sensitivity and a good reproducibility (except for Cu^{2+}), highlighted by the large number of tests performed for each compound and by the low value of standard deviation. However, it can be pointed out how heavy metals IC_{50}s are all of the same order of magnitude, probably causing a similar effect on oxygen consumption of mitochondria, whereas organic compounds IC_{50}s are very variable.

The IC_{50} data show a very good sensitivity of the method as compared to the FM80 (Bragadin and Dell'Antone, 1994), as well as compared to other common bioassays (Pickering et al., 1989; RIVM, 1999) (Table 5). In fact, a comparison between FM80 EC_{50} and FM22 IC_{50} shows the same order of magnitude for inhibition due to the heavy metals Cd, Pb, Cu, Zn, whereas EC_{50} of Ni in FM80 is one fold higher than in FM22 IC_{50}. FM22 resulted more sensitive to organic compounds, except for DDE, than FM80. Comparing our method with other common bioassays (algal–bacterial, *Daphnia magna*, fish), we have to consider that various actions are implied. For organic compounds, the FM22 test shows greater sensitivity than the bacterial

Table 4
Heavy metals determination

	Cd	Cr	Cu	Ni	Zn	Pb[a]	Fe[a]
1	<0.01	0.5	0.09	0.19	0.24	0.05	4.20
2	<0.01	<0.01	0.01	0.03	0.86	<0.03	<0.03
3	<0.01	<0.01	<0.01	0.01	1.54	<0.03	0.10
4	<0.01	<0.01	0.01	0.02	0.18	<0.03	<0.03
5	<0.01	0.06	0.17	0.23	0.86	<0.03	10.7
6	<0.01	20.6	1.40	4.72	1.51	0.105	4.93
7	0.01	0.089	<0.01	0.65	0.81	<0.03	0.05
8	<0.01	<0.01	<0.01	0.10	0.71	<0.03	<0.03
9	<0.01	0.07	0.01	0.50	0.06	<0.03	1.27
10	<0.01	0.02	0.01	0.02	0.37	<0.03	0.08
11	<0.01	<0.01	<0.01	0.02	0.13	<0.03	0.24
12	<0.01	<0.01	<0.01	0.03	0.05	<0.03	0.06
13	<0.01	0.01	<0.01	0.01	0.07	<0.03	0.06
14	<0.01	<0.01	<0.01	<0.01	0.04	<0.03	<0.03
15	<0.01	<0.01	<0.01	<0.01	0.05	<0.03	<0.03
16	<0.01	<0.01	<0.01	<0.01	0.03	<0.03	<0.03
17	<0.01	<0.01	<0.01	<0.01	0.01	<0.03	<0.03
18	<0.01	<0.01	<0.01	<0.01	0.08	<0.03	<0.03
19	<0.01	<0.01	0.07	0.080	0.08	<0.03	<0.03
20	<0.01	<0.01	<0.01	<0.01	0.01	<0.03	<0.03
21	<0.01	<0.01	<0.01	<0.01	0.03	<0.03	<0.03
22	<0.01	<0.01	<0.01	<0.01	0.03	<0.03	<0.03
23	<0.01	<0.01	<0.01	<0.01	0.03	<0.03	<0.03
24	<0.01	<0.01	<0.01	<0.01	0.03	<0.03	<0.03
25	<0.01	<0.01	<0.01	<0.01	0.01	<0.03	<0.03

Results are in mg/l; 0.01 mg/l detection limit.
[a] 0.03 mg/l limit of detection.

and algal tests and *D. magna* (except for endrin), whereas results on heavy metals are quite different. Comparing to fish tests, the FM22 resulted more sensitive for heavy metals and organic compounds except for DDE and endrin. Something interesting has to be pointed out on DMSO: it resulted in high toxicity for FM22 and its harmfulness is quite well known. However, only toxicity data on FM80 and bacterial genotoxicity test have been found in the literature despite DMSO being a widely used solvent.

Although FM22 IC_{50}s were comparable with other tests, data on reproducibility of metals made us considering that they might require a longer time of actions in order to express an evident interaction with mitochondria. Thus, we are currently working on the development of a slightly different kind of test procedure, such to increase the time during which mitochondria and metals stay in contact. Indeed, previous results, from tests performed adding EDTA to bioassay medium, have shown no metal speciation alterations, i.e. resulting in a variable metal availability.

Results of the toxicity tests on samples confirmed the possibility of using this assay in a screening field analysis. The importance of bioassay is in its sensitivity when changes in sample characteristics occur. This fact emerged, especially, in testing samples from the same factory or plant, before and after any treatment occurred. Although, in the Venice area, samples 3–4 and 6–7 show less toxic action after treatment (samples 4 and 7), results from samples 1–2, 10–11 and 12–13 show the opposite, despite a decreasing in their metal contents. Similar results are obtained for the effluents sampled in the Rovereto area. In fact, samples 14 and 15 from the Coste Creek and sample 16 from the textile factory, show high toxicity for mitochondria, despite the few metals found; sample 19 presents a high quantity of metals but a relatively low toxic action. The complex mixture of the sample can thus have toxic results that may not always be detected by chemical analysis. Nevertheless considerations regarding reproducibility in the case of metal toxicity may be applied here too. Metal content, in these samples was quite low, thus toxicity action can be triggered by organics only.

Environmental samples are often difficult to analyse in an appropriate way. Their composition is unknown and a through screening would be very expensive and time consuming. Moreover, as compounds can synergistically/antagonistically act in the environmental systems, toxicity can even not be shown by the chemical composition. The aim of the bioassay is to synthesise the complex effect of a sample on a living organism or part of it.

Table 5
Comparison of toxicity: EC_{50} for FM80, algae, bacteria, *Daphnia*, LC_{50} for fish

	FM80: EC_{50}	Bacterial–algal tests: EC_{50}	*D. magna*: EC_{50}	Fishes: LC_{50}
Zn^{2+} [a]	0.08	0.1–0.5[b]	0.025–0.1	2.3
Cr^{6+} [a]			0.62[c]	
Ni^{2+} [a]	9.10	0.36[d,e]		
Cd^{2+} [a]	0.16	0.002–0.008[f]		11
Pb^{2+} [a]	0.08	2.7[b]		
Cu^{2+} [a]	0.09		0.069[e]	0.77
Benzene[g]		525[h]	0.2	14
DMSO[g]	3.16×10^4	0.008[i]		
DDE[g]	0.79			0.24
Endrin[g]			0.0042	0.21×10^{-3}
Dichloromethane[g]		2292[h]	220[e]	99[j]
1,3-Dichlorobenzene[g]		31[b]	7.4[e]	5
1,2-Dichlorobenzene[g]	1.00	2.2[f]	0.78	5.6
Chlorobenzene[g]	3.16	33[f]	4.3	16

Data in mg/l.
[a] Pickering et al. (1989).
[b] *Scenedesmus q.*
[c] IC_{25}.
[d] *Paramecium burs.*
[e] LC_{50}.
[f] *Selenastrum c.*
[g] RIVM (1999).
[h] *Chlorella.*
[i] *Saccharomyces c.:* positive to test of mutagenity induction.
[j] EC_{50}.

Although mitochondria preparation is time consuming, their stableness under frozen storage permits their preparation only once every several months. Mitochondria from the same preparation were used up to six months not showing any change of behaviour and sensitivity. Results from calibration of this method confirmed a high sensitivity of the FM22 method, allowing the use of a −22 °C storage temperature. The advantages of this technique are the inexpensiveness of common lab materials combined with the relatively short time of screening. In fact, it takes 20 min for the analysis and little time for the data treatment, then, results can be quickly obtained. The −22 °C frozen mitochondria test is, thus, a good predictor of toxicity, easy to handle, fast and affordable.

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Alessandra Iero received her Environmental Sciences degree in 1996 from Ca' Foscari, University of Venice, Italy. Actually she is continuing the research on mitochondria as indicator of toxicity and she is undertaking researches on freshwater quality.

Sabrina Manente received her Biological Sciences degree from University of Padova, Italy. She's currently a Ph.D. student at Ca' Foscari, University of Venice, focusing on heavy metals, biomarker and bioassay and on pollution problems.

Guido Perin is associated professor of ecotoxicology at the Department of Environmental Sciences of Ca' Foscari, University of Venice, Italy. His scientific interests include problems of pollution in water and sediments, activities on freshwater sites, on Venice lagoon and on Guanabara (Brasil) lagoon.

Marcantonio Bragadin is associated professor of toxicological chemistry at the Department of Environmental Sciences of Ca' Foscari, University of Venice, Italy. His scientific interests include in vitro measures of toxicity by xenobiotics.