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# A Pilot Study of the Water Quality of the Yarra River, Victoria, Australia, Using In Vitro Techniques

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**Abstract** A pilot study was initiated to provide the first information on the recombinant receptor-reporter gene bioassay (hormonal) activity of freshwaters in Victoria. The project involved the collection of water samples from six stations on the main stem of the Yarra River in and upstream of the city of Melbourne, Australia in April 2008 and April 2009. Samples were prepared for measurement of sample toxicity using a modified photobacterium test, genotoxicity using a high-throughput luminescent *umu* test method, and human and medaka estrogen receptor (hER$\alpha$ and medER$\alpha$), retinoic acid receptor (RAR), aryl hydrocarbon receptor (AhR) and thyroid receptor (TR) assay activity using the relevant yeast-based bioassays. Most samples were only weakly or moderately toxic, with no relationship observed to location along the river. The data for 2008 suggests that at that time the Yarra River samples contained few compounds that were, in and of themselves, genotoxic. No estrogenic or thyroid, and <1 ng/L retinoic acid receptor activity was observed. AhR activity increased with progressed downstream. AhR activity was higher in April 2009 than at the same time in 2008, perhaps as a result of extensive bush fires in the catchment in the months immediately prior to sampling. About 24% of the total AhR activity observed was associated with suspended solids.

**Keywords** Recombinant receptor-reporter gene bioassay · High-throughput luminescent *umu* test · Yarra River · Victoria · Australia

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In the late 1990s, Jobling et al. (1998) reported an unusually high incidence of intersex in wild populations of the roach (*Rutilus rutilus*) in English rivers. This was arguably the first well-documented example of a widespread sexual disruption in wild populations of aquatic vertebrates, with the reproductive abnormalities being broadly consistent with exposure to hormonally active substances associated with discharges from municipal wastewater treatment plants (WWTPs). Mispagel et al. (2005) published the first measurements of estrogenic activity in Victorian WWTP discharges, reporting that estrogenic activity was detected in most samples tested [6/7 samples: <LOD—55 ng/L 17$\beta$-estradiol equivalents (EEQ)]. In a follow up survey, Mispagel et al. (2009) noted that most of the twelve WWTP effluents investigated showed estrogenic activity in the range 1–10 ng/L EEQ, with 17$\beta$-estradiol (E2) concentrations for the most part in the range 2–5 ng/L. Discharges from WWTPs may be a significant source of much of the steroidal input into many aquatic environments, but in the past few years it has been recognised that there are other sources of hormonally active contaminants within watersheds, including storm water and agricultural run-off. Although the occurrence of endocrine disrupting chemicals

(EDCs) in rivers and their impacts on aquatic wildlife have generated a significant amount of scientific and public interest internationally, there has to date been, to the best of the authors' knowledge, no information published on their occurrence in Victorian freshwater environments.

The wide structural variety of EDCs for the most part restricts the applicability single-compound chemical analysis of complex matrices, such as found in many peri-urban waterways. Several in vitro assays have been developed to screen the 'hormonal activity' of compounds in natural waters, including ligand-binding assays, recombinant receptor-reporter gene assays, assays based on the measurement of cell proliferation, and enzyme-linked immunosorbent assays (ELISA; Streck 2009; Kinnberg 2003). Recombinant receptor-reporter gene assays, such as the yeast two-hybrid bioassays used in this study, measure the activation of a receptor, and allow for quantification of hormonal activity, without having to know the precise chemical make up of the sample.

The city of Melbourne is the capital and most populous city in the state of Victoria. The city is located at the northern-most point of a large natural bay (Port Phillip Bay), with the city centre itself positioned on the estuary of the Yarra River (Fig. 1). The metropolitan area extends along the eastern and western shorelines of Port Phillip Bay, and more than 25 km inland. The Yarra River catchment is approximately 4,000 km$^2$, and is home to approximately 2 million people. In general, water quality is good in the upper catchment, but deteriorates downstream because of diffuse pollution from changed land use, particularly from agriculture and urban development (PPWRRHS 2007). In the urbanised area of the catchment, stormwater has a major impact on the river's water quality. Several small (10,000–50,000 population equivalents) WWTPs discharge treated effluent into the Yarra River (either directly or via a tributary), although most of Melbourne's domestic and industrial sewage is transferred to two large WWTPs in the south and west of the city and after treatment discharged to the ocean. In recognition of the river's importance to Melbourne, and the potential risks that EDCs pose to aquatic ecosystems, this pilot study was initiated to provide the first information on the recombinant receptor-reporter gene bioassay (hormonal) activity of the Yarra River. To that end, grab water samples were taken from selected sites on the Yarra River on two occasions. Sample analysis was subsequently conducted for toxicity using a modified photobacterium test, genotoxicity using a high-throughput luminescent *umu* test method, and human and medaka estrogen receptor (hERα and medERα), retinoic acid receptor (RAR), aryl hydrocarbon receptor (AhR), and thyroid receptor (TR) assay activity using a suite of yeast-based bioassays.

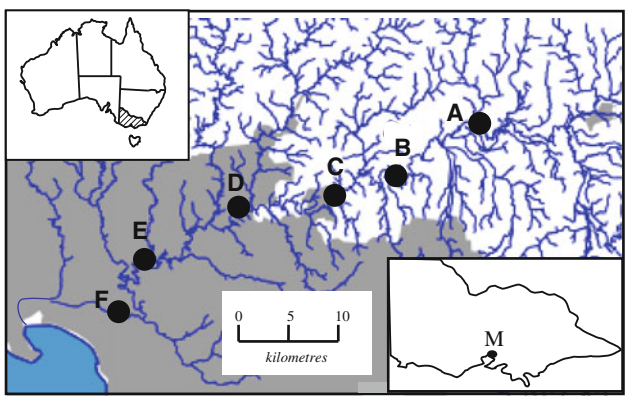


**Fig. 1** Approximate location of Melbourne (M) in Victoria, Australia, and sampling sites on the Yarra River (urban Melbourne is shaded *grey*)

## Materials and Methods

The Yarra River flows 240 km from its headwaters to the sea in Port Philip Bay. The upper sections of the Yarra River and its main tributaries (above Site A; Fig. 1) flow through forested, mountainous areas that have been reserved for water supply purposes for more than 100 years. Most of the land in the upper-middle section (between Sites C and A) has been cleared for agriculture, although there is significant peri-urban development. The land downstream of Site D (Fig. 1) is primarily urban and industrial. The lowest 10 km section of the Yarra River (e.g. at Site F) is estuarine. Water samples were collected as 'grab' or spot samples from 6 stations on the Yarra River on two occasions (April 2008 and April 2009; Fig. 1). The stations were selected for a variety of reasons: either because they were already part of Melbourne Water's Yarra River water quality monitoring program (e.g. Site E), were in a section of very high regional importance (PPWRRHS 2007; e.g. Site A), were downstream of a tributary with of very high regional importance (e.g. Site C), or downstream of a tributary with known industrial impacts (e.g. Site E).

Water samples were directly collected in glass bottles, stored on ice, and then at 4°C until processed (generally within 36 h of collection). For each site, an aliquot of the effluent (1 L) was extracted for the measurement of toxicity using a modified photobacterium test, hormonal activity using a yeast-based bioassay, and genotoxicity using a high-throughput luminescent *umu* test method. The sample preparation methods for these tests are described elsewhere (Shiraishi et al. 2000; Allinson et al. 2007, 2008), but, in short, involved filtration and adding buffer solution to the sample to ensure an acid pH (according to JEA 1998), filtration through GF/C filters to remove particulate matter, and then solid phase extraction [SPE; Octadecyl C18FF disk (Empore; 47 mm; 3 M, MN, USA)]

and elution of analytes with methanol. For the photobacterium test, the disks were eluted with methanol to provide a whole-sample extract. For the yeast-based bioassays and the high-throughput luminescent *umu* test, after evaporation the sample was re-suspended in a mixture of 3:1 hexane: dichloromethane (1 mL), and loaded onto a florisil column (Varian Bond Elut-FL, 500 mg, 3 mL; CA, USA). Thereafter, elution protocols separated the extract into three fractions, first a 3:1 hexane:dichloromethane fraction (H/D), second a 1:9 acetone:dicholoromethane fraction (A/D), and finally a methanol fraction (MeOH). The A/D fraction contained the steroid hormones, and the separation was undertaken to minimise the effects of matrix components on the bioassay systems. In 2008, where there was sufficient particulate material for testing, the GF/C disk was dried on a hotplate at 30°C, then extracted with MeOH (30 mL) by sonicating for 10 min in an ultra-sound bath. An aliquot (15 mL) of the MeOH solution was then evaporated to dryness under nitrogen, and subjected to the florisil fraction process described above.

Measurement of toxicity on whole sample extract was conducted using the method of Shiraishi et al. 1999 (described in English in Allinson et al. 2007), with data reported as the ICR50, which is a measure of how much the sample has to be concentrated to inhibit luminescence in 50% of the photobacteria. Measurement of genotoxicity was undertaken on the H/D, A/D and MeOH fractions using a high-throughput luminescent *umu* test method using *Salmonella typhimurium* TL210 strain (Nakajima et al. 2007), with and without addition of a commercially available metabolic activation system (+S9/−S9). Measurement of estrogenic and retinoic acid activity was undertaken with a yeast two-hybrid recombinant receptor-reporter gene bioassay system in accordance with the method of Shiraishi et al. 2000 (described in English in Allinson et al. 2008) using yeast cells (*Saccharomyces cerevisiae* Y190) into which the human estrogen receptor ERα or the estrogen receptor from Japanese medaka (*Oryzias latipes*) had been inserted (hERα and medERα, respectively; Nishikawa et al. 1999), and the method of Kamata et al. (2008) using the same strain of yeast into which the human RAR$\gamma$ receptor had been inserted. Measurement of AhR activity was undertaken in accordance with the method of Kamata et al. (2009) using yeast cells (YCM3) carrying the response element for the AhR complex, XRE5 (Miller 1999). Measurement of TR activity was undertaken according to Shiraishi et al. (2003).

Positive controls were used with all assays: hERα and medERα assays, 17$\beta$-estradiol and estrone (Wako Pure Chemical Industries Ltd, Osaka, Japan); RAR assay, all-trans-retinoic acid (a-t-RA; Wako Pure Chemical Industries Ltd, Osaka, Japan); AhR, $\beta$-naphthoflavone ($\beta$NF; Wako Pure Chemical Industries Ltd, Osaka, Japan); TR, triiodothyronine (T3; Wako Pure Chemical Industries Ltd, Osaka, Japan); *umu* test, 4-4-nitroquinoline-N-oxide and benzo(a)pyrene [4NQO and B(a)P; Wako Pure Chemical Industries Ltd, Osaka, Japan]. A solvent (vehicle) control dimethyl sulphoxide (DMSO, Nacalai Tesque Inc., Kyoto, Japan) was used in all cases. The agonist activities of the H/D, A/D and MeOH fractions of the sample extracts were measured, and unless otherwise stated, data from the yeast-based bioassays and the high-throughput luminescent *umu* test is reported as the sum of the activity observed in all three fractions. The bioassay method's limits of reporting (LOR) for the hERα and medERα systems were 0.1 and 0.5 ng/L 17$\beta$-estradiol equivalents (EEQ), respectively. For the RAR, AhR, and TR bioassays, they were 0.4 ng/L a-t-RAEQ, 0.4 ng/L $\beta$NFEQ, and 10 ng/L T3EQ, respectively.

## Results and Discussion

Only two sites were genotoxic without metabolic activation (-S9; Sites C and D, 2008 survey; 0.34 and 0.01 µg/L 4NQO EQ; Table 1). Notwithstanding that samples were

**Table 1** Summary of general water quality and sample toxicity data

| Site | General water quality | | | Toxicity | Genotoxicity (*umu*) | |
|---|---|---|---|---|---|---|
| | Temp. (°C) | pH | EC (µS/cm) | ICR50 | −S9 4NQO EQ | +S9 B(a)P EQ |
| 2008/2009 | | | | | | |
| (A) | 14.0/15.3 | 6.3/6.1 | 83/93 | 179/400 | <0.02/– | <0.2/– |
| (B) | 15.1/17.9 | 6.4/6.4 | 128/146 | 295/219 | <0.04/– | <0.3/– |
| (C) | 14.4/17.1 | 6.4/6.5 | 171/180 | 328/214 | 0.34/– | <0.9/– |
| (D) | 15.8/16.4 | 6.4/6.8 | 160/214 | 178/199 | 0.01/– | <0.3/– |
| (E) | 17.4/17.6 | 6.9/6.6 | 190/246 | 216/170 | <0.02/– | <0.4/– |
| (F) | 19.0/18.4 | 6.4/7.4 | 19,000/32,400 | 72/173 | <0.02/– | <0.6/– |

–, no sample; *umu* test toxicity in µg/L

collected on a single day in 2008, the data for that year suggests that at that time the Yarra River samples contained few compounds that were, in and of themselves, genotoxic. No sites were genotoxic with metabolic activation (+S9). Incubating the sample with metabolic activation system (+S9) before challenging the assay provides, in broad terms, an indication of the relative proportions of readily metabolisable and more recalcitrant compounds contributing to genotoxicity, and whether there are chemicals present in the sample whose biodegradation products are significantly more genotoxic than parent compounds. However, practically it is difficult to compare the *umu* test response −S9 and +S9, in part because different positive controls are used, and in part because the limits of reporting for the test +S9 are much higher than tests without metabolic activation. Moreover, since it is difficult to normalise the test to absolute luminescence due to luminescence quenching by proteins, and because there are some compounds that produce a positive response −S9 but a weaker response +S9, it should be considered that the *umu* test −S9 and +S9 is effectively evaluating different groups of compounds. Unfortunately, we were unable to assess genotoxicity in 2009 and thus assess the potential impact of the extensive bush fires in the catchment in the months immediately prior to sampling.

Sample toxicity was measured using a modified photobacterium test. In part, toxicity data was acquired as part of recombinant receptor-reporter gene bioassay quality assurance processes, to ensure that a 'no response' from an assay results from 'no activity' rather than the response from the bioassay having been compromised by direct sample toxicity. With this photobacterium test system, the lower the ICR50 reported, the higher the toxicity of the sample, with samples considered highly toxic to the photobacterium at an ICR50 < 100, and non-toxic at an ICR50 > 400. In this context, in 2008, most of the Yarra River samples were considered to be only moderately-weakly toxic (Table 1). Only Site F exhibited high toxicity (in 2008 only). Although the reasons for the observed high toxicity are as yet unknown, there are several possible influences including roadside run-off from the nearby highway and a large stormwater drain that enters the Yarra River at Site F which often has poor water quality (*Pers. Comm.* Dr. Vincent Pettigrove, Melbourne Water). Similar sample toxicity was observed in 2009, although in this case toxicity appeared to increase from upstream to downstream (Table 1).

No estrogenic activity was observed in the dissolved phase of samples collected from the main stem of the Yarra River in either 2008 or 2009 (Table 2). The results of the positive controls run at the same time as the samples suggest the bioassays were performing to expectation, and thus capable of measuring receptor activity at low concentration, e.g. <2 ng/L EEQ. Consequently, in conjunction with toxicity data, lack of response from any the bioassay is assumed to be due to lack of receptor-inducing compounds in the samples, and not, for instance, due to a toxic effect on the yeast cells. It is natural to wonder how these results compare with those found elsewhere, although it is at times risky to do so because differences between studies (e.g. scale, scope, techniques used) can make the comparisons difficult. In this context, the lack of estrogenic activity is consistent with observations in Queensland using the E-screen assay (<0.2–0.95 ng/L EEQ; Leusch et al. 2010), and in six rivers of central Japan (<0.3 ng/L EEQ using the same bioassays as this pilot study; Inoue et al. 2009).

**Table 2** Summary of observed recombinant receptor-reporter gene bioassay activity (dissolved phase)

| Site | Receptor activity | | | | |
|---|---|---|---|---|---|
| | EEQ | | RAR | TR | AhR |
| | hERα | medERα | a-t-RA EQ | T3 EQ | βNF EQ |
| 2008/2009 | | | | | |
| (A) | <0.1/<0.1 | <0.4/<0.4 | <0.4/<0.4 | –/<10 | 10/19 |
| (B) | <0.1/<0.1 | <0.4/<0.4 | <0.4/<0.4 | –/<10 | 11/23 |
| (C) | <0.1/<0.1 | <0.4/<0.4 | <0.4/<0.4 | –/<10 | 14/20 |
| (D) | <0.1/<0.1 | <0.4/<0.4 | <0.4/<0.4 | –/<10 | 20/27 |
| (E) | <0.1/<0.1 | <0.4/<0.4 | 0.88/<0.4 | –/<10 | 25/23 |
| (F) | <0.1/<0.1 | <0.4/<0.4 | <0.4/<0.4 | –/<10 | 19/27 |

–, no sample; all measured parameters in ng/L

Only one site on the Yarra River (Site E; 2008) was positive for retinoid activity (0.88 ng/L a-t-RAEQ), which is consistent with the RAR agonist activity reported by Inoue et al. (2010) in samples collected from 20 sites on the Ina and Lake Biwa–Yodo Rivers in central Japan (1.1–23.5 ng/L a-t-RA EQ). If we assume that the RAR agonist behaviour of all the RAR ligands in our samples is additive and can be represented by the toxicity of a-t-RA, then we can qualitatively report that in this study the observed RAR activity is at least three orders of magnitude smaller than the concentration of retinoic acid isomers observed to produce developmental impacts, e.g. post-embryonic malformations of the Japanese flounder (*Paralichthys olivaceus*; ~7,500 ng/L a-t-RA; Haga et al. 2002).

No thyroid activity was observed in the dissolved phase of samples collected from the main stem of the Yarra River in either 2008 or 2009 (Table 2), which is also consistent with Jugan et al. (2009), who reported no TR agonist activity in river water extract at sites on Seine River, France, upstream of any WWTP influence, and Inoue et al. (2009) who reported little TR activity in six rivers of central Japan except at two sites downstream of WWTPs.

All samples were positive for AhR activity, which increased as sampling progressed downstream (Table 2). AhR activity was generally marginally higher in April 2009 than at the same time in 2008, perhaps as a result of the bush fires that swept through the upper part of the catchment in the months prior to sampling. Bioassays based on the binding to the AhR have not previously been used in Victoria, and there is limited information on the AhR activity of rivers elsewhere. Moreover, it is difficult to compare these results directly with the few other studies reporting AhR activity in river waters because of differences in bioassay systems incubation protocols, cellular type (e.g. yeast cells cf. human cell lines), expression of response (e.g. chemiluminescence cf. luciferase expression) and reporting units (e.g. $\beta$NF equivalents cf. 2,3,7,8-TCDD (TCDD) equivalents). However, with respect to the latter, Kamata et al. (2009) have suggested that the AhR affinity of $\beta$NF is about the same as that of TCDD in the YCM3 cell assay, especially at the relatively low concentrations that are significant for data analysis in this assay. Therefore, the numerical activities of the river waters samples relative to TCDD are roughly the same as reported for $\beta$NF. Consequently, even though TCDD EQ activity was not assessed directly (for laboratory safety reasons), we are able to say that this study's results are two to three orders of magnitude higher than those of Rawson et al. (2009), who observed up to 0.032 ng/L TCDD EQ in water samples from 10 wetlands and 24 creeks and rivers in Sydney using the H4IIE bioassay. Moreover, if we assume that the AhR agonist behaviour of all the AhR ligands in our samples is additive and can be represented by the effects of 2,3,7,8-TCDD, the observed AhR activity (16–279 ng/L $\beta$NF EQ) would represent concentrations of 2,3,7,8-TCDD that would be at least an order of magnitude lower than those reported to cause jaw malformation in zebra fish embryos via AhR-mediated down-regulation of the chondrogenic transcription factor, sox9b (Xiong et al. 2008; 1 μg/L).

Suspended solids in the water column may be a considerable sink for some hormonally active, hydrophobic compounds. In this study, AhR activity was observed in suspended solids extracts for almost all samples (Fig. 2). There was a good correlation between AhR activity observed in the dissolved phase and that of suspended solids extracts ($r^2 = 0.58$), with overall approximately 24% of the total AhR activity associated with suspended solids. Unfortunately, we were unable to assess the AhR activity of suspended solids in 2009 and thus cannot assess the potential impact of the extensive bush fires in the catchment in the months immediately prior to sampling on the AhR activity of suspended solids. The risk to aquatic organisms, particularly benthic animals, may be significantly underestimated by ignoring the activity of suspended solids.

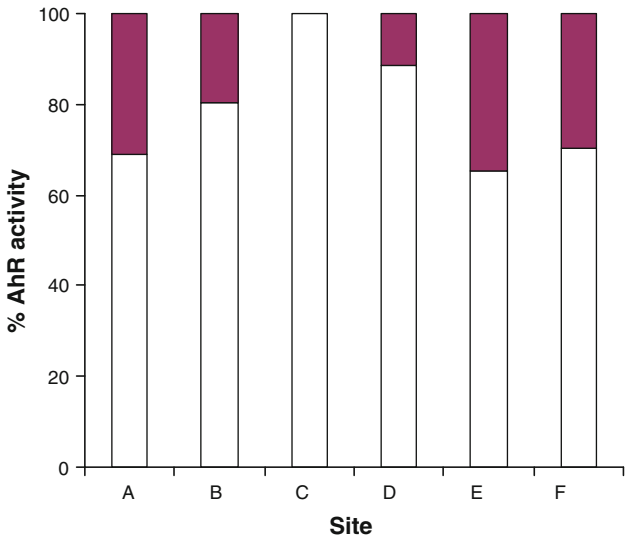


**Fig. 2** Relative distribution of AhR activity (*open square* dissolved phase, *filled square* suspended solids)

The causal chemicals inducing AhR activity were not identified in this pilot study. Consequently there is a need to further investigate the occurrence of hormonally active and genotoxic compounds in the river water, suspended solids and sediments, and to assess the ecological significance of the data in the context of land use within the catchment.

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