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Contents

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Summary

Water samples taken from three popular swimming holes in Kakadu National Park during periods of high visitation were screened for oestrogenic activity using the Yeast Estrogen Screen (YES). The signals detected were compared to levels in samples taken upstream of the swimming sites, in addition to those reported from impacted sites overseas and to the levels shown to cause whole organism effects in laboratory studies. Detectable but low levels of oestrogenicity were found in all Kakadu plunge pools sampled. Plunge pool water oestrogenicities were found to be similar to upstream sampling sites, lower than in most samples reported in the literature and lower than levels shown to cause reproductive effects in whole organisms. As such, it was concluded that the biota in Kakadu plunge pools are at low risk of endocrinological disruption from oestrogenic compounds potentially introduced by swimmers.

Introduction

Endocrine disruption

All animals rely on chemical messengers (hormones) to control bodily processes such as development, growth, maturation, reproduction and homeostasis (Tortora & Anagnostakos 1987; deFur et al 1999). An increase in the number of cases of developmental and reproductive impairment reported in overseas wildlife populations has sparked intense research into the potential for chemical contaminants to disrupt normal endocrine function in animals. Over the past decade, scientists have accumulated compelling evidence relating these population level impacts to the exposure of animals to elevated concentrations of particular chemicals (Kookana et al 2003).

An endocrine disrupting compound (EDC) has been defined as ‘any exogenous compound that causes adverse health effects in an intact organism or it’s progeny consequent to changes in endocrine (hormonal) function’ (Campbell & Hutchinson 1998). EDCs can act in a number of ways to induce a response in a whole organism. Some compounds have been shown to mimic natural hormones (thereby producing similar responses to them), while others can block their action (Chapman & Moore 2003). Others can affect glands or the central nervous system and consequently inhibit hormone production and release (Kookana et al 2003).

A vast range of chemicals have been implicated as endocrine disruptors including pesticides, plastisicers, natural and synthetic oestrogens (in sewage effluent), plant sterols, pharmaceuticals, surfactants and heavy metals (Jobling & Sumpter 1993; Stahlschmidt-Allner et al 1997; Smaglik 1998; Lim et al 2000; Choe et al 2003), while many still remain untested. Aquatic organisms, in particular, are at risk of exposure to EDCs with many waterways receiving wastewaters from industrial, urban and agricultural sources.

Examples of morphological and behavioural effects of exposure to EDCs in whole organisms are readily found in the literature. A classic example is the observation of gonadal and penile abnormalities in the American alligator (*Alligator mississippiensis*) and subsequent linkage to agricultural runoff into a Florida Lake (Guillette et al 1994, 1996). Howell et al (1980) attributed the masculinisation of female mosquitofish in a wild population to an exposure to high concentrations of β-sitosterol, a naturally occurring plant compound in a pulp paper mill effluent, while Petrovic et al (2002) observed male fish with both testicular and ovarian tissue downstream of a Spanish sewage treatment plant. Laboratory studies have demonstrated that exposure to concentrations of 100 ng/L and above of 17β-estradiol (natural oestrogen) significantly reduced the gonopodial length (modified anal fin for sperm transfer) along with male-female approaches and copulatory attempts in mosquitofish (Doyle et al 2002). Relating this information to population level effects in wildlife, however, is more difficult because of a paucity of baseline ecological information (Campbell & Hutchinson, 1998).

To date, no firm relationship has been established between the structure of known EDCs and their hormonal activity (Vinggaard et al 1999). In addition, many EDCs act at very low concentrations (Smaglick 1998) or are unidentified in a sample (Chapman & Moore 2003) making it necessary to use biological screening, rather than, or in conjunction with chemical analysis to assess the potential effects of contaminated waters to organisms.

Biological screening

International workgroups are currently developing 'full suite' methodologies for the biological assessment of endocrine disruptors. The general concensus is that a combination of laboratory and field studies are required to identify and fully understand the risks of particular contaminants. Laboratory testing is essential to distinguish EDC effects from other factors in the field (by identifying a direct cause and effect at either the molecular or whole organism level) (deFur et al 1999), while follow up field studies are required to determine that the effects observed in the laboratory are a true indication of ecological disturbance (eg the masculanisation of female gastropods does not necessarily interfere with their reproductive ability (Shahara et al 1998)).

In vitro and *in vivo* laboratory experiments have proven useful for screening environmental samples for endocrine activity (Chapman & Moore 2003) and provide a 'warning light' that a particular sample/contaminant should be further examined (Vinggaard et al 1999). They have the advantage over chemical characterisation in that they integrate the overall potency of a sample, including the inactive compounds, along with those that act agonistically or antagonistically, and they account for additive and nonadditive interactions (Villeneuve et al 2000). For these reasons they are particularly useful when the constituents of a sample are unknown.

In vivo tests are considered to be better models to investigate the effects of EDCs than *in vitro* tests because they account for a range of different mechanisms and complex interactions that are involved in the elicitation of a physiological response in a whole organism (Gillesby & Zacharewski 1998). A limited number of short-term *in vivo* tests, for example the uterotrophic and vaginal cell cornification assay, are available overseas, however, because they were developed to investigate human health issues, their ability to to accurately identify and assess environmental samples has been questioned (Zacharewski 1998). Longer term tests based on more ecologically relevant endpoints such as vitellogenin (yolk-precursor protein) production in fish and are relatively time-consuming and expensive, involving the exposure of whole organisms for periods of up to weeks. This approach to testing has normally been taken for sites already identified as being impacted by pollutants. With the lack of any evidence that

EDCs were actually an issue in Kakadu plunge pools it was considered premature to initiate large scale testing such as this when short-term *in vitro* tests were available for a first-pass screening assessment.

In vitro tests utilise a particular cellular mechanism, eg. receptor binding, gene expression or enzyme activity, that responds when exposed to a particular hormone mimic. One such screen is the Yeast Estrogen Screen (YES) that was developed and validated by Routledge and Sumpter (1996).

This test relies on oestrogenic compounds present in a sample binding with a human oestrogen receptor that has been genetically inserted into a yeast cell (figure 1). An expression plasmid is also contained in the cell so that upon binding, a reporter gene Lac-Z is produced. Lac-Z encodes the enzyme β-galactosidase, which passes through the yeast cell membrane into the surrounding liquid media where it metabolises a chromogenic substrate. The colour change in the substrate from (yellow to red) can be measured photometrically, thus providing a quantitative measure of the oestrogenic activity (ng of estradiol equivalents) of a sample (Rehmann et al 1999). The major disadvantage of using this screen in isolation is that as it is an oestrogen receptor-mediated screen and will only detect oestrogen mimics in a sample. Androgenic compounds and substances that can have toxic effects on the central nervous system, or glands, may go undetected. In addition, specific receptor mediated screens such as the YES do not account for the influence of absorption, metabolism, distribution, and excretion on the manifestation of their activity (Shelby et al 1996, Giesy et al 2002). All of these factors will influence the degree of effect in a whole organism and the failure to detect a signal using a receptor mediated screen will result in a false negative. Despite this, short term laboratory tests such as the YES provide rapid, sensitive and biologically relevant responses at a small expense (Chapman & Moore 2003). Provided that the test limitations are understood, then valuable information into the potential of a sample to cause endocrine disfunction can be obtained. For the purposes of this preliminary study it was decided that the most appropriate approach was to use this quantitative and well validated *in vitro* test and compare our results to the literature.

Kakadu National Park plunge pool study

With an average of 165,000 visitors to Kakadu National Park (KNP) each year, managing the impact of people is a high priority for the Traditional Owners and Parks Australia North (PAN). The national park is situated 180 km from Darwin, in the wet-dry tropics of Northern Australia (figure 2). The distinct wet and dry seasons greatly influence the numbers of visitors to the park, with the majority of tourists choosing to explore Kakadu during the months of April to September when sites are easily accessible. Many people choose to swim in waterfall plunge pools throughout the park, with sites such as Gubara, Gunlom and Maguk receiving particularly high numbers of visitors at peak times.

Many of these visitors wear sunscreens, deodorants and other cosmetics that wash off into the water and may act as endocrine disruptors. It is also likely that people are urinating in these pools while swimming. Other research, on sewage effluent (Aldercreutz et al 1986, Ternes et al 1999), indicates that it is possible for natural and synthetic oestrogens to be introduced into the water from the urine of females, particularly those on the contraceptive pill.

The potential for ecological impacts of sunscreen was brought to the attention of Parks Australia North by Dr. Mika Peck from the University of Sussex, England. Mika had undertaken part of his PhD studies at ***eriss*** in Jabiru making him familiar with tourist locations within Kakadu. His concerns were based on new research where some sunscreen

ingredients had been shown to exhibit oestrogenic activity (Mueller et al 2003, Schlumpf et al 2001, Schreurs et al 2002).

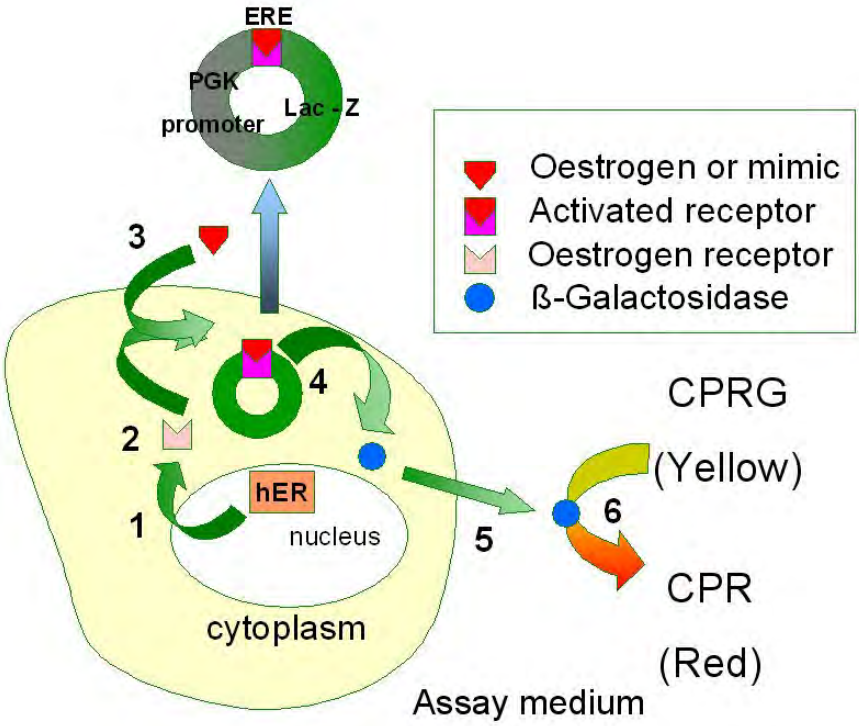


Figure 1 Schematic of the oestrogen inducible expression system in yeast

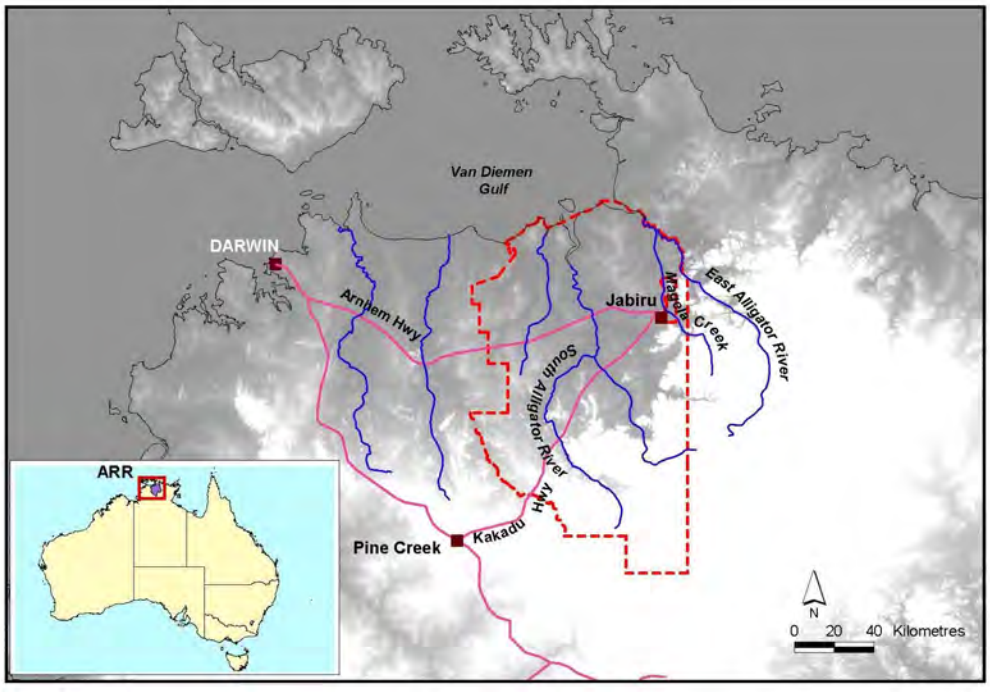


Figure 2 The location of Kakadu National Park within the wet-dry tropics of northern Australia

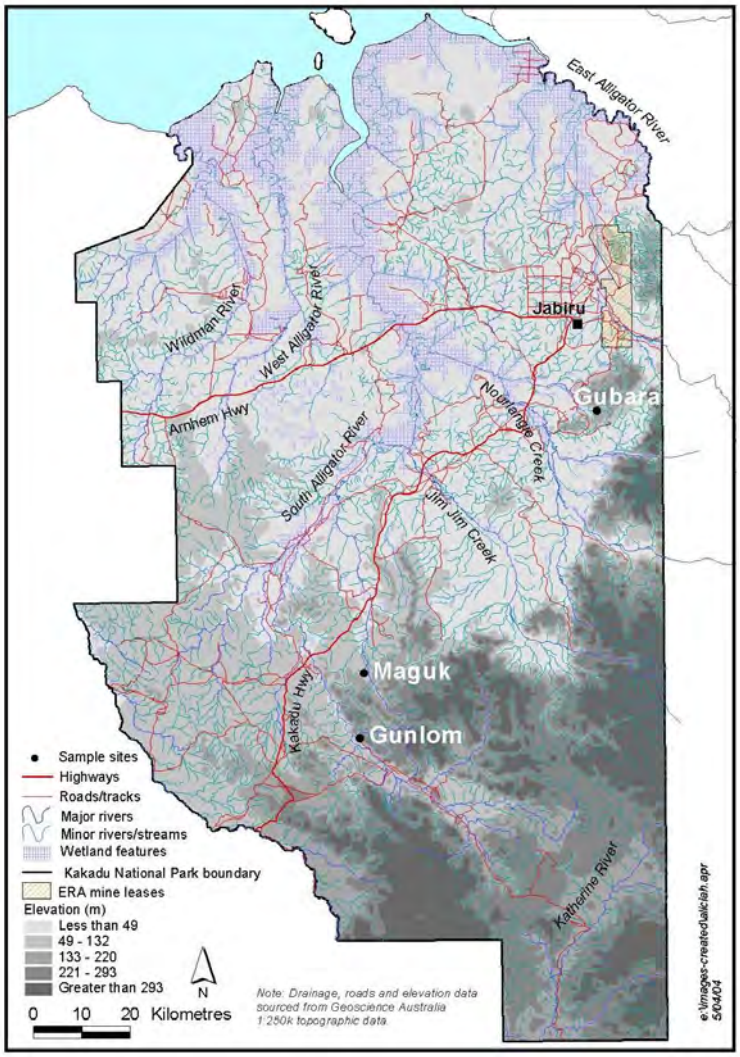
PAN approached ***eriss*** ecotoxicology staff for advice and, with verification from Australian scientists working in this field (C. Doyle and R. Lim) that a potential issue existed, it was decided that a joint preliminary study should be undertaken to assess the potential for endocrinological impacts from tourists swimming in Kakadu plunge pools. It was agreed that PAN and ***eriss*** staff would choose sampling sites and times and undertake sampling together. ***eriss*** staff would extract the samples using solid phase extraction and the YES screen would be undertaken at the University of Sussex.

Aim

The aim of this study was to determine whether Kakadu plunge pool water exhibited oestrogenic activity and if so, to assess the potential ecological effects by comparing the levels observed to those in the current literature.

Materials and methods

Sampling sites and times



Based on park records (traffic counters), recommendations from rangers and personal observation of visitor numbers, three locations, Gubara, Maguk and Gunlom were identified as the most likely to be contaminated by EDCs (figure 3).

The aim at each location was to take water samples from the pools receiving high numbers of swimmers and, where possible, to take comparative samples upstream of each site at a location that received minimal visitation (ie as reference sites).

Figure 3 Location of the three sampling sites Gubara, Gunlom and Maguk within Kakadu National Park

Gubara

Gubara differed from the other two locations in that it is more popular in the wet season when most other tourist sites are inaccessible and the pools are flowing. It is also the only site where comparative samples could be taken under both high and low stream flow conditions (wet and dry seasons). As such, two sampling times were considered appropriate for this site. The first trip was undertaken in late February 2003 (high visitation and high stream flow) and the second in late May 2003 (high visitation and low flow). Two samples were taken in February from:

1) the 'Garden of Eden' waterfall swimming pool (figure 4a);

2) upstream of the 'Garden of Eden' pool (reference).

In May, the upstream site (2) was completely dry and could not be sampled. The previously fast flowing main channel of the creek had slowed to a gently flowing pool that was being used as a swimming hole by tourists, hence this was included as a third site in the May sampling trip;

3) the swimming pool in the main creek channel (figure 4b).

Single samples were taken at each site in February and three replicate samples in May.

Figure 4 Gubara sampling sites. a: Site 1, 'Garden of Eden' pool. b: Site 3, main creek channel pool

Gunlom

Using historical traffic counter data to estimate a time when visitation was still high yet stream flow had subsided, a sampling date of late August was chosen for Gunlom. Two sites were sampled in triplicate:

1) the shallow sandy swimming area at the bottom of the falls (figure 5a);

2) the main swimming pool at the top of the falls (figure 5b).

It was intended that a third sample be taken from the shallow sandy pool behind the cascade at the top of the falls as a comparative reference site, however, there were many footprints around the pool indicating that numerous people had swum here. The creek upstream of this pool was dry which made a reference site at this location unattainable.

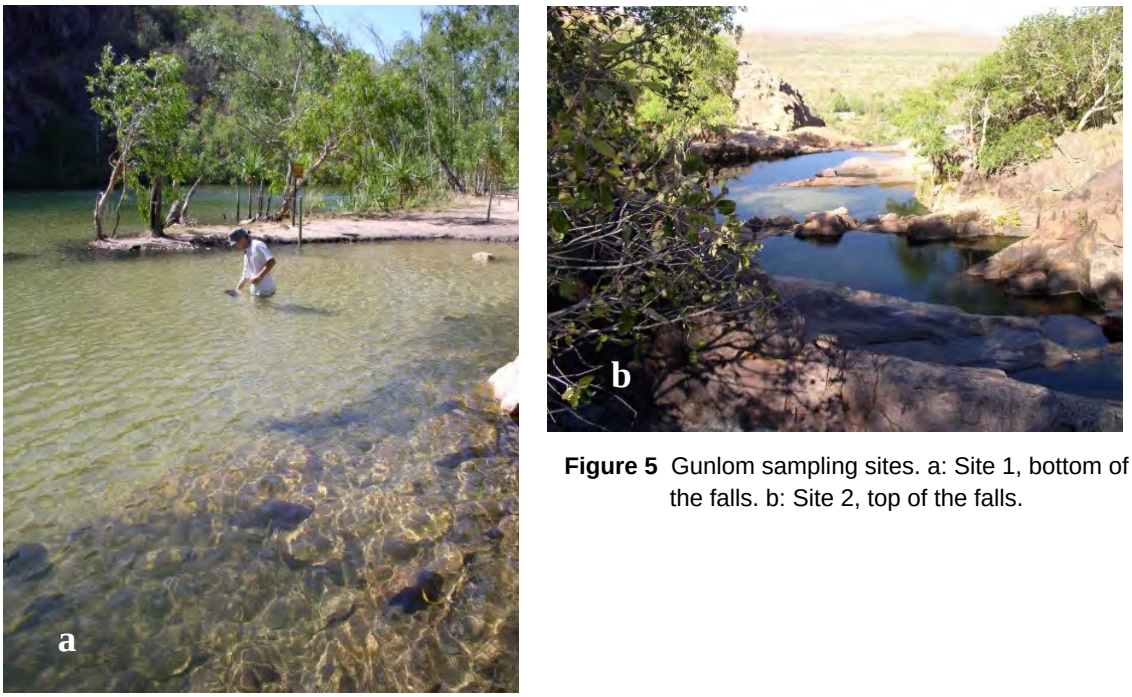


Figure 5 Gunlom sampling sites. a: Site 1, bottom of the falls. b: Site 2, top of the falls.

Maguk

Traffic counter data for Maguk indicated that late September was the most appropriate time for sample collection. Three sites were sampled in triplicate:

1) the entry-point for swimmers in the lower plunge-pool (figures 6a and b);
2) the most downstream swimming pool above the falls (figure 6b);
3) further upstream through the rocky arch where the gorge opens up to a shallow creek (reference).

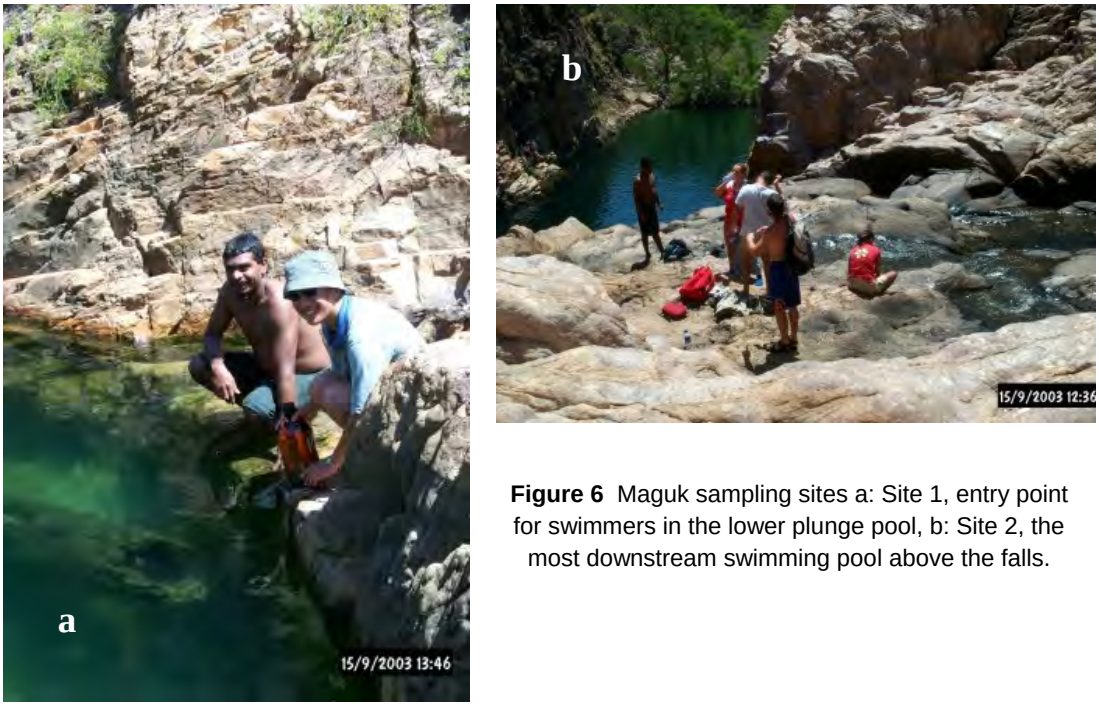


Figure 6 Maguk sampling sites a: Site 1, entry point for swimmers in the lower plunge pool, b: Site 2, the most downstream swimming pool above the falls.

Sampling equipment preparation

Amber glass Winchester bottles (2.5 L), 250 mL glass Schott bottles and 10 mL measuring cylinders were prepared by soaking in a 5% concentrated nitric acid bath for 24 h. On removal from the acid, bottles were hand rinsed with Elix water (reverse osmosis and cation exchange, Millipore, Molsheim, France) prior to undergoing a detergent (Gallay, Clean A non-phosphate powder, Gallay Scientific, Burwood, Victoria, Australia) wash and two Elix rinses in a laboratory dishwasher. Bottles were allowed to dry by evaporation, rinsed twice with analytical grade methanol, allowed to dry again and capped for storage.

Water sampling

Winchester bottles were rinsed three times with sample water and filled to the neck (2.5 L) by taking half of the sample from the surface of the water body (considering the potential hydrophobic nature of the chemicals of concern) and the second half from 15 cm under the surface. The samples were acidified by adding 2.5 mL of glacial acetic acid and capped with an aluminium foil lining between the sample and the plastic screw-on lids. Samples were placed in a slurry of ice within 1.5 h of collection and transported to the laboratory within 6 h of collection.

Water sample pre-treatment

Water sample pre-treatment enables pre-concentration of the sample, resulting in a lower final detection limit during screening (Tanghe et al 1999). In this study, a very low assay limit of quantitation[1] of 36 pg/L was achieved through this process.

On arrival at the laboratory, each sample was flushed with nitrogen for 1 min and stored in an ice slurry overnight (0°C). Samples were extracted the following day by solid phase extraction (Waters Oasis HLB, 12 cc, 500 mg columns) at a rate of less than 20 mL/min. For samples collected during the May 2003 sampling trip this was done by hand, using 60 mL syringes soaked in Milli-Q water (Millipore, Molsheim, France). However, due to the highly laborious nature of this method and the need for triplicate replication, a vacuum manifold was built to enable the sample to be connected and left to elute through the columns independently (figure 7). The manifold was checked regularly to check flow rates and to ensure that air was not pulled through the columns after the sample had passed through. The columns were then removed, packed into a plastic bag, flushed with nitrogen, sealed and stored in the refrigerator at < 4°C. The exact volumes extracted were measured (by marking the level on the bottle before extracting) and recorded. Columns were packed on ice and express posted to the University of Sussex, England within 5 days of sample collection.

Blanks were prepared for each sample collection trip and were analysed alongside the plunge pool water samples to determine if there was any contamination introduced in the collection, storage, pre-treatment or screening stages. Blanks were prepared in the laboratory by adding 2.5 L of Milli-Q water to clean Winchester bottles and adding acetic acid, storing in an ice slurry, flushing with nitrogen and extracting through SPE columns as described for the samples.

Upon arrival at the University of Sussex, solid phase cartridges were eluted with two 5ml solvent rinses of methanol, one of 5ml dicloromethane and one of 5ml hexane. The solvents

[1] Above the limit all values can be accurately quantified however, below this estrogenicity can be detected and quantified at a lower level of confidence.

were combined and evaporated to dryness then made up to 1 ml in ethanol and stored at -80ºC until testing on the YES assay.

Figure 7 Vacuum manifold apparatus used to pass plunge pool water samples through solid phase extraction columns

Sediment sample collection

Studies have demonstrated the ability of river sediments to act as a sink for the accumulation of endocrine disrupting chemicals at concentrations up to 750 times that measured in the overlying water (Peck et al 2004). Accordingly, one sediment sample was collected from each site on each sampling trip. Samples were collected using a stainless steel gardening trowel and placed into clean 250 mL Schott bottles. Any overlying water was decanted by gently tipping the bottle allowing the water to trickle out. An aluminium foil liner was placed over the neck of the bottle which was then capped. The samples were placed in an ice slurry within 1.5 h of collection and transported to the laboratory within 6 h of collection. On arrival, the samples were flushed with nitrogen, frozen then express posted to the University of Sussex with the water sample extraction columns. Silt dominated sediments were targeted during sampling due to their greater contaminant holding capacity, although all samples were relatively sandy as is the nature of the plunge pool sediments.

Sediment sample preparation

All Glassware was cleaned by detergent washing followed by rinsing in ultrapure water (UHQ, Elga, Bucks, UK) then baking for 12 hours at 500ºC to ensure removal of all organic compounds. Sediments were homogenised and 1g added to 15 ml glass vials. The following series of ultrasonic analytical grade solvent extractions (Microson XL, Heat Systems, Farmingdale, NY) was carried out: 2.5ml methanol, 5ml dichloromethane and 5ml hexane. After each extraction step the sample was centrifuged and the supernatant transferred to a 5ml test tube and evaporated to dryness. The extraction series was recombined and made up to 1ml in ethanol.

Yeast Estrogen Screen

Extracts of samples and blanks were added in a series of dilutions to a test multiwell plate, and the ethanol was allowed to evaporate at room temperature. Concentrations of E2 were analyzed in parallel as a positive control. Yeast and assay medium containing the chromogenic substrate were added to the wells, and the plate was incubated for 3 d. The absorbance of each sample was determined, corrected for untreated controls and yeast growth, and compared with that of the E2 standard. The estrogenicity measured in water and sediment samples was analysed as described below and expressed as equivalent E2 values (EEQs) (Peck et al 2004).

Data analysis

The oestrogenicity was calculated by determining the EC_{50} of the sample curve then determining the mean of the two points straddling the EC_{50} absorbance. The estradiol equivalent concentration was then calculated by reference to the standard estradiol curve. The relative measurement of oestrogenicity was determined by calculating estradiol equivalency factors (EEQs), that relate the potency of a particular compound or sample to the oestrogenicity of 17β-estradiol. For example, DDT is reported to have 0.000001 times the potency of 17β-estradiol, therefore 1 000 000 ng (i.e. 1 mg) of DDT would be required to elicit the same effect as 1 ng 17β-estradiol (Chapman and Moore 2003).

Results and discussion

Quality assurance

A blank signal of 176 pg/L EEQ (Figure 8a) in the first collection trip was considered unacceptably high and was addressed by re-cleaning the collection and screening glassware and by replicating future samples. Subsequent blank signals were all found to be below 13.5 pg/L EEQ which is well within acceptable levels based on the precision of the screen.

Variability was high between replicates at several sites with standard deviations at two sites exceeding the mean recorded oestrogenicities. For this reason, results were not tested for significant differences between sites. The high variability experienced was attributed to a number of factors including the sampling method, the chemical nature of potential EDCs, and/or the precision of the screening method.

The chosen sampling method (taking three separate 2.5 L grab samples, consisting of 50% water skimmed from the surface and 50% from approximately 15 cm below the surface) was based on the conjecture that potential EDCs, particularly from sunscreens, may form a slick on the surface microlayer of the water. As this was a preliminary study, and the number of samples that could be processed was limited, a composite sample such as this was thought to provide the best opportunity of detecting EDCs. It is possible though, that by collecting from the surface layer, that the probability of collecting from a more concentrated ‘patch’ was increased. Another scenario is that these compounds may not be evenly mixed in the water bodies making reproducible replicate samples difficult to achieve. In addition, the level of precision attainable in the YES assay close to its limit of detection has been reported to give unexplained variability of up to 40 pg/L EEQ (Mika Peck pers. comm.) which is similar to the degree of variability experienced at most sites.

Variability at the pg/L level was not considered to interfere in the conclusiveness of this study because, as discussed in the following section, the oestrogenic activity observed is around two orders of magnitude lower than what is generally observed at impacted sites internationally.

Screening outcomes

Water samples

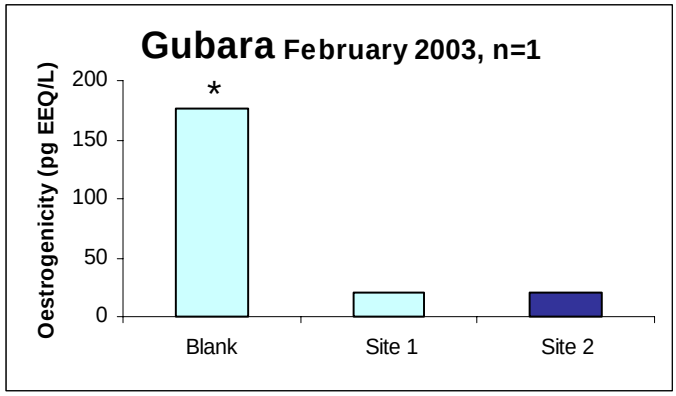
All samples exhibited extremely low levels of oestrogenicity ranging from 9 - 130 pg of oestradiol equivalents per litre (EEQ/L) (figure 8 a-d). These values are far lower than those reported in the literature for YES screens undertaken on effluent samples and receiving waters in the Northern Hemisphere. Typical oestrogenic activities in a number of sewage treatment effluents ranged between 0.35 - 218 ng EEQ/L (Desbrow et al 1998, Murk et al 2002, Tanghe et al 1999, Thomas et al 2001) and between 179 - 389 ng EEQ/L in chemical and food processing effluents (Tanghe et al 1999). Three out of five Belgian river waters screened were found not to be significantly different from the controls (equivalent detection limits were not given), however, the remaining two rivers gave signals ranging between 32-34 ng EEQ/L. These results are still more oestrogenic (by a factor of 100) than our highest signal. Thomas et al (2001) described two samples taken up- and downstream of a treated sewage outlet with respective EEQs of 0.3 and 0.5 ng/L as having 'little activity' and reported previous studies in the United Kingdom where the EEQs at other sewage treatment works averaged around 50 ng EEQ/L. Further, more recent work on two UK rivers, above and below treated effluent inputs, showed levels at less than 0.04 ng/L, with effluent inputs at up to 2.9 ng/L. The low levels below effluents was explained by rapid absorption to sediment and degradation.

In order to address how environmental levels of oestrogenicity such as these will affect the reproduction and development of whole organisms, a number of studies (primarily using fish) have attempted to identify concentrations of EDCs that elicit reproductive-related responses over longer timeframes. Kramer et al (1998) showed that egg production in fathead minnows was reduced by more than 50% at 120 ng/L 17β-estradiol after a 19 day exposure. While the lowest concentrations found in the literature by Murk et al (2002) to stimulate vitellogenin (egg protein pre-cursor) production in male fish were 0.3 ng/L EEQ (28 week exposure, Sheahan et al 1994) and 10 ng/L EEQ (3 week exposure, Routledge et al 1998).

More recently, Doyle et al (2002) found that exposure to 20 ng/L 17β-estradiol over an 84 day period had no effect on gonopodium length or the mating behaviour of male mosquitofish. Concentrations of 100 and 500 ng/L 17β-estradiol, however, did result in significant reductions in both of these variables.

In this study, the two sites receiving low visitation (shaded dark in figure 8) were found to give signals of a similar magnitude to those that have high visitation (pale shading). This indicated that the signals detected represent the natural background oestrogenicity of the near-pristine waters coming from the sandstone country. It has been widely reported that various groups of natural oestrogenic substances are produced by plants (eg isoflaonoids, flavones, chalcones and lignanes) and fungi (eg zearalenone) (Korner et al 2001, Chapman & Moore 2003) that may enter waterways in vegetated areas.

From the current available literature it appears that oestrogenicities in the low pg/L range, as detected in the Kakadu plunge pool samples, are not considered to be a risk to aquatic organisms, although it must be noted that not all studies tested low enough concentrations to produce 'no effect' data.



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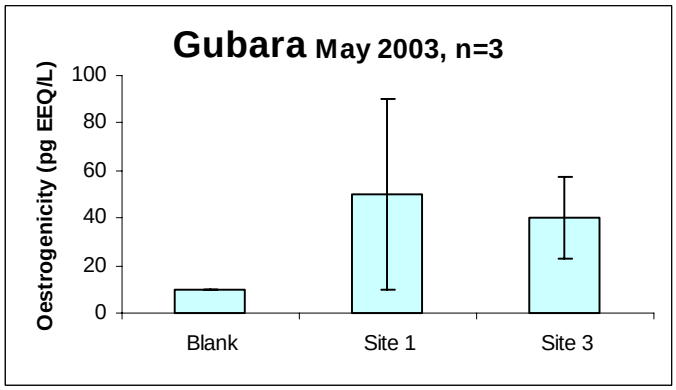

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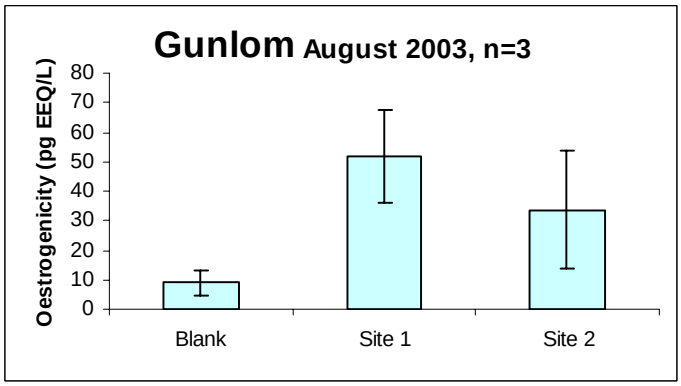

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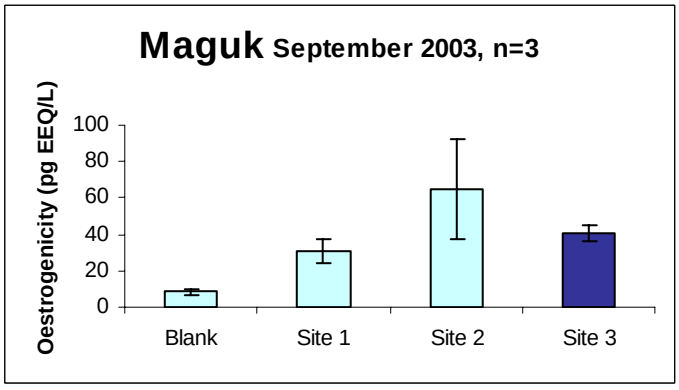

d)

Figure 8 Oestrogenicity of Kakadu plunge pools sampled during periods of high visitation. Dark coloured columns represent samples taken upstream of swimming holes (ie. control sites). Error bars indicate the standard error of the mean. * identifies a contaminated sample.

Sediment samples

Oestrogenicity was not detected in any of the sediment samples (<80 pg/kg – limit of detection). This was not surprising considering that levels in the overlying water were very low and that the samples consisted mostly of sand, which has a much lower adsorptive capacity than siltier sediments.

Conclusions and recommendations

Detectable but low levels of oestrogenicity were found in all Kakadu plunge pools sampled using yeast estrogen screening methodology. In comparison to international literature the values reported here were 6 - 6000 times lower than in European wastewaters and 1 - 530 times lower than in ambient surface waters. To date no studies have demonstrated endocrine disruptive effects at the oestrogenic activities reported for the swimming holes indicating a very low risk to aquatic biota. Further to this, the oestrogenicity of upstream sites, that were known to receive low visitation, was similar to that measured in samples from popular swimming holes. This indicated that the low levels detected were representative of the natural background oestrogenicity of the local waters.

It should be noted, however, that this is a very new field of science where methodologies are still being developed and refined. The YES method used in this study is one of the more validated and widely used protocols but does have recognised limitations (which are discussed in detail in the introduction of this report). For this reason, the general consensus is that a heirachical suite of *in vitro*, *in vivo* and field studies should be undertaken in order to make a solid conclusion on the potential for long-term ecological effects resulting from EDCs. It is therefore recommended that should other methodologies become more affordable and readily available in Australia that further sampling should be considered for swimming holes where EDC contamination is likely. This would be particularly relevant for swimming holes where visitation levels increase significantly.

Acknowledgments

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