Review of Bioassays for Monitoring Fate and Transport of Estrogenic Endocrine Disrupting Compounds in Water

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ABSTRACT

Endocrine disrupting compounds (EDCs) are recognized contaminants threatening water quality. Despite efforts in source identification, few strategies exist for characterization or treatment of this environmental pollution. Given that there are numerous EDCs that can negatively affect humans and wildlife, general screening techniques like bioassays and biosensors provide an essential rapid and intensive analysis capacity. Commonly applied bioassays include the ELISA and YES assays, but promising technologies include ER-CALUX™, ELRA, Endotect™, RIANA, and IR-bioamplification. Two biosensors, Endotect™ and RIANA, are field portable using non-cellular biological detection strategies. Environmental management of EDCs in water requires integration of biosensors and bioassays for monitoring and assessment.

1.0 Introduction to Endocrine Disrupting Compounds

Endocrine disrupting compounds (EDCs) are chemicals with the potential to cause negative effects on endocrine systems of humans and wildlife. A wide array of natural and synthetic chemical compounds have been identified to elicit estrogenic response including pharmaceuticals, pesticides, industrial chemicals, and heavy metals (Giesy et al., 2002). The U.S. Environmental Protection Agency (EPA) defines an EDC as:

> an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior. (USEPA, 1997, pg 1)

This broad class of chemicals includes both natural and synthetic estrogens (xenoestrogens such as oestrogens and pseudoestrogens). Specific examples of estrogenic EDCs include: pesticides like atrazine, organochlorines, dieldrin, and toxaphene (Hayes et al., 2002; Ramamoorthy et al., 1997; Arnold et al. 1996a), surfactants such a alkyphenol-ethoxalates and nonylphenols (Folmar et al., 2002; Legler et al., 2002a), pharmaceutical estrogens (17β-estradiol, 17α-Ethynlestradiol, etc.) (Legler et al., 2002a; Folmar et al., 2000), as well as, other industrial compounds like PCBs, bisphenols, and dioxins (Howdeshell et al., 1999; Ramamoorthy et al., 1997; Mocarelli et al., 1996).

It is inevitable that more EDCs will be identified with time and there are mounting problems with monitoring and managing this form of environmental pollution (Petrović et al., 2004). These problems arise from the extremely low concentrations of EDCs that elicit effects, and also because the magnitude and impact of the effect will depend on not only concentration, but also timing of release, and the dynamics of the ecosystem. Research continues to focus on (1) environmental toxicology of these compounds under various exposure regimes, (2) identifying potential sources to contaminated water-bodies, and (3) analytical and bioassay

methods to detect specific compounds or classes of compounds. However, large-scale (regional) monitoring, treatment, and water management approaches to minimize the environmental impacts of EDCs remain to be developed. Environmental management solutions for water contamination most often rely on source mitigation, discharge timing and quantity control, and low cost treatment systems. Only source mitigation (*e.g.* removal of alkyphenol-ethoxalates from pesticide formulations) and EDC treatment in conventional wastewater systems (*e.g.* Johnson and Sumpter, 2001) have been considered in the literature. Therefore, research in EDC monitoring, fate, and transport is still needed.

This review will detail the state-of-the-art for monitoring EDCs in environmental waters from the perspective of water resources engineering and summarize the issue of EDC contamination in the environment while providing an introduction to many of the bioassays and biosensors available. A few new technologies have emerged that may offer a greater capacity to monitor and mange EDC concentrations in water. Examples of recent bioassays results from surface water and water waters discharge locations in California, USA are provided for discussion.

1.1 Background on EDCs

Given that many of the EDCs identified have the potential to cause an estrogenic response at very low concentrations, parts per billion to parts per trillion, it is troubling that organic wastewater contaminants were found in 80% of 139 surface water streams sampled across 30 states in the US (Kolpin et al., 2002). Moreover, measurable concentrations of many of the EDCs mentioned above have been found in wastewater, surface waters, sediments, groundwater, and even drinking water (Petrović et al., 2004; Benfenati et al., 2003, Petrović et

al., 2003; Snyder et al., 2003). Wastewater treatment plants have been studied as a major source for EDCs (Snyder et al., 2003; Legler et al., 2002a).

As pointed out in Brown et al., (2001) it is often difficult to provide direct mechanistic connections between observed EDC concentrations and actual endocrine disruption in a wildlife population. Studies have demonstrated some form of endocrine disruption in many different species including fish, reptiles, birds, mammals, alligators, marine gastropods, and snapping turtles, (Jimenez, 1997, Table 2). These effects varied and included developmental deformities, changes in fecundity, and decreases in hatching to immunological effects and cancer. Bowerman et al. (2000) suggested that population level effects of hormone disrupting chemicals have been associated with reproductive and teratogenic effects observed in the bald eagle population within the Great Lakes Basin. In the same region, lake trout exposure to dioxin, TCDD, and related compounds has been attributed to endocrine disruption (McMaster, 2001). Feminization of males has been observed in wild leopard frogs, *Rana pipiens* (Hayes et al., 2002) and wild carp, *Cyprinus carpio* (Sole et al., 2003) living in environments known to have elevated levels of EDCs.

Human exposure to EDCs is a critical concern. Measurable concentrations of the EDC, nonylphenol (NP) was found in all of the 60 different common food products sampled in a study in Germany (Guenther et al., 2002). NP has also been found in Tokyo Bay, Japan where EDCs have been implicated as a potential cause of observed decreases in male sperm count in the human population (Isobe et al., 2001). Human tissues demonstrated to be sensitive to estrogens, through estrogen receptor expression, include the brain, immune system, cardiovascular system, lungs, mammary glands, liver, kidneys, reproductive tract (ovaries, testes, uterus, prostrate), adipose tissue, and bone (Müller, 2004). The largest known exposure of humans to elevated

EDC levels was the result of an accidental release of dioxin (TCDD) near Seveso, Italy. Nearly 20 years after the spill, increased instances of cancer, reproductive, and immunological problems have been observed in the population (Baccarelli et al., 2002).

2.0 Bioassays and Biosensors for Endocrine Disruptors

The recent recognition of the dangers of EDCs has promoted the development of analytical methods, including HPLC, GC/MS, and LC/MS/MS (Petrović et al., 2002; Huang and Sedlak, 2001, Petrović and Barceló, 2000). While these techniques provide the necessary sensitivity, accuracy, and precision for EDC monitoring, they measure EDCs individually and do not give information on biological response or synergistic effects. These analytical techniques require trained personnel, specialized analytical equipment, preconcentration steps, and are not easily adapted to rapid, intensive or real-time monitoring.

Bioassays and biosensors provide alternative detection methods to traditional laboratory analyses. Bioassays are defined as methods that use biological materials with a mode of detection, but require an external mode of quantitation such as a microscope for cell counts, luminometer for bioluminescence, or a multimeter for voltage. Biosensors will be used here to refer to self-contained EDC detection systems including both a mode of detection and mode of quantitation in the same device. Detection in a bioassay or biosensor occurs by a number of mechanisms, for example some biosystems detect ligand binding, while others depend on immune response to detection EDCs. Both bioassays and biosensors may provide either a qualitative or quantitative response. Cell proliferation in response to EDCs, for example, may always increase in a bioassay, but not always at a consistent level. Such a bioassay would be qualitative. On the other hand, if a consistent relationship exists between the bioluminescence intensity from a bioassay and EDC concentration, then this technique is quantitative.

A major advantage of biosensors is in pre-screening and tests can be performed in real-time, at remote sites, on multiple samples, and can use the sample water directly without preconcentration (Rodriguez-Mozaz et al., 2004a). In addition, many bioassays have the potential to be developed into biosensors. A summary tree of the bioassays and biosensors, as well as their mode of detection and mode of expression is presented in Figure 1 that relates to all the techniques discussed in the following sections.

Bioassays and biosensors may be based on either whole cells, like yeast or cancer cells, or biological materials like estrogen receptor ligands or enzymes applied in systems without cells. The following discussion organized these techniques by whole organism, cell and non-cellular assays. A comprehensive summary of compound-specific bioassay studies along with the mode of estrogenic activity is provided in Table 2 of Giesy et al., (2002). A number of *in vivo* and *in vitro* bioassays have been developed as qualitative indicators of estrogenic activity. *In vivo* assays are exposure studies of whole organisms in the environment contaminated with EDCs. In some cases bioassays are sensitive to concentrations lower than analytical detection limits. Procedures have also been developed to determine the *in vivo* effect of estrogens and xenoestrogens on entire organisms (Legler et al. 2002a; Hamers et al. 2001; Folmar et al. 2000). These studies can be costly, using measures of reproduction, growth, sperm count, gonad development, or other sexual development (Jimenez, 1997). *In vivo* assays also commonly require autopsy of organisms to assess the response to EDCs. While this approach may more directly assess the affects of EDCs on organisms, it is usually not well suited for routine water quality monitoring.

In vivo studies to assess exposure to an EDC are necessary to determine realistic environmental impacts, however, controlled *in vitro* methods are needed to routinely monitor the

presence of EDCs in the environment. Numerous *in vitro* assays for EDCs have been developed recently (Soto et al. 1995). *In vitro* assays examine response of estrogen receptors, cell proliferation, gene transcription or vitellogenin production with exposure to EDC contaminated samples (Jimenez, 1997), but do not allow conclusions about the total impact on whole organisms (Müller, 2004).

Two estrogen receptors (ER) have been identified that serve as the initial points of activation for estrogenic effects in humans and animals, providing the foundation for many *in vitro* biosensors. These estrogen receptors are hER-α, which is well known, and hER-β, which is more recently characterized, where the "h" in this case denotes human origin. These ERs either trigger an associated estrogen response element (ERE) to bind with DNA, modulating transcription of target genes, and thereby causing a measurable immunoassay response to the estrogen (Lascombe et al., 2000) or the ER binding (ligand-binding) itself induces a measurable response (Seifert, 2004). The following sections will discuss a few examples of bioassays.

2.1 In Vivo Whole Organism Assays

Negative effects of EDCs have been observed in amphibians, fish, and insects that may be used as biological indicators of EDC pollution in aquatic environments. Frog populations have been suggested to be particularly sensitive to endocrine disrupting compounds in the environment. Gonadal abnormalities have been observed in 10 to 92 percent of male wild leopard frogs (*Rana pipiens*) examined from throughout the United States (Hayes et al., 2002). In fact, new technologies for *in vitro* EDC quantification have returned to locations where frog deformities have been observed to analyze estrogenicity (Erb et al., 2001).

Many fish assays for estrogens have been developed by the US EPA and others using rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), sheephead

minnow (*Cyprinodon variegates*) and zebrafish (*Brachydanio rerio*) (Legler et al., 2002a; Fenner-Crisp et al., 2000; Folmar et al., 2000). There are various approaches for determining estrogenic response in these organisms including deformities, reproductive deficiencies, egg and offspring development, and serum proteins like vitellogenin. Vitellogenin is a yolk protein in female fish liver produced in response to estrogens that can be extracted from plasma and measured (Jimenez, 1997). In addition, transgenic zebrafish have been bioengineered with luciferase expression coordinated to vitellogenin production causing estrogen exposed fish to emit luminescence (Legler et al., 2002a)

2.2 In Vitro Single Cell Bioassays

Various *in vitro* bioassays exist, usually relying on transgenic bioengineered cells (yeast or breast cancer cells) that connect an estrogen receptor from humans or more a sensitive fish like trout, to express a measurable response. These bioassays may be broadly categorized as (1) ligand-binding assays where an ER is connected to a promoter that produces a measurable luminescence or colormetric response (2) immunoassays where the ERE to bind with DNA, modulating transcription of target genes causing a measurable response, and (3) cell proliferation assays where the number of cells in indicative of estrogenicity (Scrimshaw and Lester, 2004). The early bioassays include E-SCREEN (cell proliferation response), YES (colometric response), and ER binding assays (luminescent response) (Fang et al., 2000). Comparisons of these approaches demonstrate that results are not exactly the same, but are reasonably correlated (r^2-values of 0.78 and 0.85) (Fang et al., 2000). Gu et al., (2002) developed a biosensor using recombinant *E. coli* containing the *luxCDABE* luminescent expression from *Vibrio fischeri* to assess the toxicity of many EDCs. Many of the estrogenic compounds, including NP, bisphenol A, and pesticides, were demonstrated to cause various types of toxic response. This study

demonstrates the necessity for establishing both estrogenic, antiestrogenic, and toxic biosensor responses to EDCs, as antiestrogenicity and toxicity inhibit expression of the *lux* (luminescent) and *β-galactosidase* (colometric). Each of these *in vitro* bioassays is described in the following sections.

E-Screen

This cell-proliferation bioassay generates more cells in the presence of estrogen that correlates to estrogen concentrations in a sample (Soto et al. 1995). MCF-7 breast cancer cells are exposed to both positive (17β-estradiol) and negative (no estrogens) controls, as well as to samples potentially containing estrogenic compounds. The comparison of the total cell proliferation to the positive control provides the basis for demonstrating estrogenic response.

YES

The Yeast Estrogen Screen or YES cells are engineered with a human estrogen receptor, which binds to an estrogen response element regulated-expression plasmid (lac-Z) coded to express *β-galactosidase* (Arnold et al., 1996). This enzyme reacts with a substrate in the culture media to release chlorophenol red. The intensity of the colometric response can be quantified using a spectrometer at specific light absorbance wavelength peaks at 420 and 600 nm (Legler et al., 2002b). In an application of the YES assay, the investigators observed combined additive estrogenicity with the presence of multiple estrogenic compounds, demonstrating the need for total screening tools that are not compound specific (Silva et al., 2002).

ER-CALUX

This genetically engineered, commercially available biosensor, the **E**strogen **R**esponsive **C**hemically **A**ctivated **LU**ciferase e**X**pression (**ER-CALUX®**) assay (BioDetection Systems, Amsterdam, The Netherlands) has been developed using the T47D human breast

adenocarcinoma cell engineered to express an estrogen receptor (luciferase). The luciferase will luminesce when exposed to a chemical by lysing the cells and adding the substrate luciferin (Legler et al., 2002b). Essentially, this bioassay produces an organic light-emitting compound when exposed to estrogen. The light emission or bioluminescence can be quantitatively measured using a luminometer.

IR-bio-amplification Analysis

Only one whole-cell bioassay has been proposed that does not require genetic engineering or cell proliferation counting. IR-bio-amplification is a technique developed at the Lawrence Berkeley National Laboratory (LBNL) that is based on synchrotron radiation (SR)-based Fourier transform infrared (FTIR) spectromicroscopy (Holman et al., 2000). The basic idea is that changes in light diffraction can be related to changes in molecules with living cells. In order to obtain the resolution necessary to discern changes in cells, a highly focused light source is required. The infrared spectromicroscopy facility on Beamline 1.4.3 at the Advanced Light Source (ALS) at LBNL has been used as the light source for the IR-bio-amplification. Mid-infrared light is low in energy, so it is nondestructive to biological materials, allowing the detection of subtle intracellular changes in live cells as they are exposed to environmental stimuli like EDCs (Holman, et al., 2000). The diffraction of the light is detected at 128 individual sensors and the response is calibrated to measurement of a normal functioning cell. Cell response must be documented for various life stages of an EDC sensitive cell to define the "background" light diffraction pattern. Once this background is defined, a change in cell response due to exposure to EDCs may be tested.

2.2.1 Comparison of Bioassays

Although Fang et al., (2000) found reasonable correlations between bioassays, there is the potential for bioassay results to differ either in the magnitude of response or even produce an opposite response. Ramamoorthy et al. (1997) in attempting to recreate the original study of Arnold et al. (1996a) observed different synergistic associations between weakly estrogenic pesticides. McLachlan et al., (1997) noted in their response to the comment note from Ramamoorthy et al. (1997) that,

> It is difficult to compare the results of the study by Ramamoorthy *et al*. to ours because the assays they used, while appearing to be similar to ours, were in each case different. (Pg. 405).

The authors (McLachlan et al., 1997) were recognizing that different bioassays potentially produce different estrogenic responses to the same EDC. A comparison of the YES and ER-CALUX assays not only revealed a different magnitude for response (ER-CALUX was 20 times more sensitive), but also found that the ER-CALUX assay did not find estrogenic activity for carboxylate acid derivatives of NPEOs (NP1EC and NP2EC) (Legler et al., 2002b). These ECDs have been demonstrated to have significant estrogenic activities in other studies (Routledge and Sumpter, 1996; White et al., 1994). Moreover, Legler et al., (2002b) found butylbenzylphthalate to be a antiestrognic in ER-CALUX which is in disagreement with the YES and E-SCREEN assays (Harris et al., 1997; Jobling et al., 1995; Soto et al., 1995). Pesticides like atrazine were also not found to be estrogenic in ER-CALUX (Legler et al., 2002b), which conflicts with results of Hayes et al. (2002). Such differences are often attributed to cell membrane permeability and cell life functions (Legler et al., 2002b).

2.3 In Vitro Biological Receptor/Ligand Binding Based Biosensors and Quantitative Bioassays

If cell membrane permeability and life stage are to blame for the estrogenic measurement of bioassays, the potential next step is to determine if bioassays may be developed without cell

membranes. *In vitro* estrogen receptor based biosensors are an active area of current research. Bioassays that do not require whole cells avoid difficulties relating to membrane permeability, cell function, organism life stages, and toxicity responses to a given water sample. Some quantitative bioassays like the Enzyme-Linked Immunosorbent Assays (ELISA) and the Enzyme Linked Receptor Assay (ELRA) still require laboratory detection systems, but provide a measure of EDC concentration. Biosensors like the Endotect™, the RIver ANAlyser (RIANA) and Biacore™ systems have the potential to be made field portable. A description with recent applications of these biosensors is provided in the following section.

ELISA

Enzyme-Linked Immunosorbent Assays (ELISA) tests are currently available for many of the environmentally relevant surfactants and estrogen compounds, as well as pesticides, antibiotics, and other personal care products (Gascón et al., 1997). ELISA techniques have been developed for various media including water, blood serum, urine, and sediments (Sun et al., 2001; Gascon et al., 1997; Oubina et al., 1997). Immunoassays quantify the biological response to an estrogen by causing the estrogen receptor to activate a response element. They have been applied to environmental monitoring for many years, particularly for pesticide distribution and timing in river discharges (Thurman et al., 1992). A magnetic particle-based solid-phase ELISA for pesticide analysis was compared to GC-MS analytical techniques by Gascon et al., (1995). However, solid phase fluorescence and colormetric immunoassays have also been developed and compared to mass spectrometry techniques (Huang and Sedlak. 2001; Bretcht et al., 1998, Gascon et al., 1997).

ELRA

A competitive ligand binding receptor assay approach similar to ELISA has been developed that employs receptor binding indicating a biological effect of agonism or antagonism (Garrett et al., 1999; Seifert et al., 1999). This assay is more specific than ELISA, as the ligand binding activates the measurable response as opposed to an immunological response. The enzyme-linked receptor assay (ELRA) has been successfully applied to environmental samples and also developed into a biosensor (see Biacore™ below). A luminescent ELRA has also been developed and compared to YES assay revealing a linear correlation (Seifert, 2004). This article also pointed out that while many bioassays are reported to provide more sensitive detection, that the ELRA provide an analytical as opposed to a relative estrogenic activity.

Endotect™

A new biosensor has been developed based on human estrogen receptors (hER) connected to a promoter that produces fluorescence measured in an evanescence-type detector (Erb et al., 2001). The biosensor portion s\of the Endotect™ (ThreeFold Sensors, Ann Arbor, MI) , the hER and promoter, are attached to an optical fiber that measures a total fluorescent response down the fiber length (not at the end of the fiber) in the process of evanescence. The Endotect™ is commercially available as a field portable, hand-held device with refills for the reagent and evanescent optical fibers. This biosensor has also been successfully field tested (Erb et al., 2001), however, a comparison to other techniques has not yet been published.

RIANA

The RIver ANAlysis (RIANA) is a multi-analyte immunosensor that uses total internal reflection fluorescence to determine the atrazine, isoproturon, and estrone levels in water (Rodriguez-Mozaz et al., 2004b). This system uses a HE-NE laser excitation source for fluorescently labeled antibodies that are specifically bound to analyte derivatives. In a manner

similar to the Endotect™ system, an evanescent field is produced around an optical fiber that may be quantitatively measured. Initial testing of the RIANA is very promising with clear determination of the 3 target analytes, low variability, and a demonstrated ability to measure the analytes in various water sources including river water, groundwater, and wastewater (Rodriguez-Mozaz et al., 2004b). Moreover, concentrations measured by the biosensor showed a linear agreement with LC-MS measurements.

Biacore™ Surface Plasmon Resonance

A surface plasmon resonance device is sold by Biacore Company (Piscataway, NJ, USA) that can be applied to measure the mass of estrogens in samples. This technology uses light refraction from a sensor chip with a very thin gold layer to measure molecules interacting with biological receptors on the sensor chip. Microfluidic systems carry the sample solutions over the senor chip, where estrogenic compounds bind with specific ligands, and then the optical detection system measures the plasmon resonance which can be related to concentration of the xenoestrogens. A plasmon resonance biosensor of for xenoestrogens has been developed by Usami et al. (2002).

A similar surface plasmon resonance Biosensor was developed using the Biacore™ system in combination with both the receptor assay ELRA and the immunoassay ELISA (Seifert et al., 1999; Hock et al., 2002). The ELISA based biosensor successfully measured concentrations of the pesticide atrazine, with a detection limit of 0.2 μg/L.

Other Potential Biosensors

Electrochemical biosensor approaches for EDC detection have been proposed by Zhihong et al., (1999) and Murata et al. (2001). Zhihong et al. (1999) examined a piezoelectric sandwich-type assay using an estrogen response element immobilized in the biosensor. The binding of

17β-estradiol was detected with a lower limit of 2.2 μg/L. Another potential biosensor uses a histidine-tag fusion system, where the histidine-tag interacts with a Ni(II) chelate adsorbant, the author found an estrogen concentration dependent voltammetric response (Murata et al., 2001). This result suggests that an electrochemical biosensors related to estrogenic binding to a human estrogen receptor are possible.

More recently a fluorescent indicator that can discriminate between estrogen agonists and antagonists was developed by Awais et al., (2004). It was demonstrated that the fluorescent indicator could be applied to living cells and the dose-dependent florescent response measured to determine estrogenic activity in cells. This indicator approach, called the Single Cell Coactivator Recruitment (SCCoR), has the potential to make target cells of many different species into biosensors.

3.0 EDC Fate and Transport with Bioassays

While wastewater treatment facilities have been demonstrated to be sources for EDC in numerous studies (Legler et al. 2002a; Sumpter, 1998; Sumpter, 1995), applications of bioassays for downstream source characterization have been somewhat limited. A majority of EDC source and distribution studies have collected samples that were analyzed in the lab using protocols developed for HPLC, GC/MS, and LC/MS/MS techniques (Rice et al., 2003; Petrović et al., 2002; Ferguson et al., 2001). Screening the large number of samples required for fate and transport characterization, however, would be more efficient using bioassays and biosensors. Using screening results to evaluate the presence and impacts of EDCs, more targeted investigations may be used to identify the compounds involved and their degradation, fate and transport in that environment. However, successful application of biosensors in the field can be

a complicated engineering problem and research is still needed to transform laboratory bioassays into portable field biosensors.

Both Petrović et al., (2004) and Heisterkamp et al., (2004) have proposed a targeted analysis or bioassay-directed chemical analysis (BDCA) approach in which initial screening is performed to correlate estrogenic activity found in the YES assay with specific chemical species measured by LC-MS, GC-MS, or LC-MS-MS detection. This approach is similar to the US EPA's toxicity, identification, and evaluation (TIE) often required in regulatory discharge permits after toxicity is demonstrated in a permitted discharge. Many studies have already combined chemical and biological monitoring (Aerni et al., 2004), however using BDCA under development and still largely laboratory based. The potential to use such a BDCA in the field to select and transport bioassay-identified samples back to the laboratory would be an excellent advantage.

The ultimate goal for a monitoring system is to provide information at the temporal and spatial resolution that characterizes the fate and transport of a target compound. A representation of the transfer and partitioning of EDCs into different compartments is shown in Figure 2. EDCs are potentially released into the environment through wastewater treatment discharges, surface nonpoint source runoff, and atmospheric deposition of particulates and aerosols. Many EDCs have moderate to high organic partitioning coefficients (K_{oc}), so the mass that does not remain soluble often ends up in organic complexes in sediments and on colloids (Table 1). In the sediments there is the potential for biological uptake, degradation and transformation to less mobile and more mobile forms. If mobilized, the EDC complexes may move back into the water column or downward toward groundwater. Therefore exposure pathways exist for humans and wildlife consuming either water or biomass. Biosensors may not perform well in all the

necessary media including wastewater, sediments, or biological materials, however, the spatial and temporal resolution from a reliable biosensor could focus investigations on a compartment where EDC mass has partitioned. Then the more sensitive laboratory techniques may be performed on fewer samples of similar media. Biosensors could greatly improve EDC monitoring schemes and aid in the development of environmental management solutions.

Other chemical and physical properties of some common EDCs are also listed in Table 1. The distribution coefficients of EDCs between the aqueous and solid phases (K_{oc} values) in relation to the local concentration of organic carbon are listed as Log K_{oc} in Table 1. The range of LogK_{oc} values would suggest that these EDCs sorb to organic carbon and are relatively immobile. Moreover, the solubility values would suggest that most EDCs would generally not remain in solution. However, the EDCs in this Table have been identified in water samples collected throughout the world (Petrović et al., 2004; Peré-Trepat et al., 2004; Rice et al., 2003; Stachel et al., 2003; Ying et al., 2001; Ferguson et al., 2001; Thurman et al., 1992). In some cases EDCs have been found in groundwater and drinking water samples suggesting some type of soluble transport (Lopez-Roldan et al., 2004; Petrović et al. 2003). Possible explanations for these observations include (1) more soluble precursors experienced transport, (2) colloid facilitated transport, and (3) enhanced solubility through elevated pH (many EDCs have a pK_a around 10) and the formation of micelles. Longer chain nonylphenol ethoxylates can have critical micelle formation concentrations (CMC) of 4.25×10^{-5} mg/L (Brix et al., 2001).

Screening for xenoestrogens will often express estrogenic potency in relation to an estrogen like estradiol. The estrogenic effect measured by the bioassay divided by the measured estrogenic activity of estradiol at the same concentration is the estradiol equivalent factor (EEF). Then the total concentration of an estrogenic EDC multiplied by its EEF would be the estradiol

equivalence (EEQ) factor (Giesy et al., 2002; Legler et al., 2002a). Some examples of EEFs may be seen in Table 1. An assessment of estrogenicity in sediments collected from marine locations throughout The Netherlands using the ER-CALUX assay found EEQs ranging from 4.5 to 38.4 (Legler et al., 2002b). Given the affinity of EDC to sorb to sediments, these EEQ demonstrate the potential for accumulative estrogenic potential in sediments.

3.1 Applications of Bioassays and Biosensors

Some biosensor technologies have attempted to create field bioassay systems or field portable biosensor including the RIANA and Endotect™ systems. However, there are few examples of estrogenic screening of water samples using ether bioassays or biosensor. Those that could be found are summarized in the following sections, however the authors expect this to be an active area of future research.

Applications of whole cell bioassays as a screening tool for estrogenicity have largely been performed with the YES assay. The YES bioassay was applied, along with other methods, to examine the persistence and degradation of estrogenic hormones in soils (Colucci et al., 2001; Colucci and Topp, 2001). The YES assay results of estrogenicity over time agreed reasonably well with degradation rates monitored using radioactive carbon labeled 17β-estradiol. These authors found rapid degradation of estrogenic hormones (17β-estradiol, estrone, and 17α-ethynylestradiol), decreasing estrogenic response and immobilization of these compounds close to background levels within 60 days (Colucci et al., 2001; Colucci and Topp, 2001). Other studies of transport through soils have been performed in lysimeters, with sewage sludge and EDC mixtures applied at the surface (Dizer et al., 2002). This investigation found measurable estrogenic response in effluent from 30 and 90 cm depth and suggested that a fast mobilization may have occurred due to the soluble fraction and colloid facilitated transport.

The authors of this review are currently gathering field samples to apply the ELISA technique to screen river water and wastewater treatment influent and effluent for estrogens. Results showing levels of three estrogen compounds found in the Sacramento River, up and downstream from a wastewater treatment plant are in Table 2. Results for estradiol and testosterone analyses in the influent and effluent of two non-conventional wastewater treatment plants are in Table 3. While these results are preliminary, they demonstrate potential for estrogenic compounds to enter and exit municipal water treatment systems discharging into surface waterways.

A synthesis of a large data set (including 32 different geographic locations) on EDCs available for coastal and harbor waters and sediment in Spain was attempted in Peré-Trepat et al., (2004). Statistical analyses including principal components analysis and a multivariate curve resolution using alternative least squares method were applied the data set to identify relationships between measured EDCs and sources. The study found that the geographic location of the EDC source could be reasonably identified using three principle components for water samples and four for sediment samples. Interestingly the study concluded that, although EDC "hot spots" could be generally identified using these techniques, the over all distribution of EDCs suggested ubiquitous sources (Peré-Trepat et al., 2004). This study demonstrates the potential for nonpoint sources of EDC and that control (through regulation) of point source discharges from wastewater treatment plants or industrial sources could be insufficient to reduce EDC to below active levels in water and sediment. As mentioned at the beginning of this review if source control is not a complete solution, then management solutions must rely on adjusting mixing and discharge timing or EDC treatment technologies.

4.0 Future Outlook and Research Needs

It was the objective of this review to summarize available bioassays and biosensors for estrogenic endocrine disrupting compounds (EDCs) from the perspective of application for environmental monitoring. Greater detail on each of these assays may be found in the references provided. A comparison of the various bioassays reveals that while the most commonly applied approaches are the ELISA and YES assays, there are many promising technologies available including ER-CALUX, ELRA, Endotect™, RIANA, and IR-bioamplification. There does appear to be some comparability problems for estrogenic activity measurement made using different bioassays and one suggestion is that cell and membrane permeability plays a role in limiting estrogen receptor exposure to EDCs. This suggests that the ligand based and immumoassay based approaches not dependent on cell function may be preferable. This is also true if the ultimate goal is to produce a field portable assay, where maintaining health microbes in field conditions may be problematic. The two promising field portable biosensors the Endotect™ and RIANA have both use biological detection strategies without whole cell bioassays. Other approaches that appear to have future potential as field portable assays are the IR-bioamplification and electrochemical biosensors.

The major advantage provided by biosensors for estrogenic activity is the capacity to estimate the cumulative EDC effects in an environment. The direct relationship between *in vitro* bioassays and *in vivo* effects on aquatic organisms and wildlife is a continuing area of research. However, the advantage of using a bioassay a screening tool in a bioassay-directed chemical analysis (BDCA) or toxicity, identification, and evaluation (TIE) approach is great. The impossibility of analyzing samples for all the possible known EDCs, even neglecting unknown EDCs, necessitates the BDCA or TIE approach. In addition to screening for sources and directing more detailed analyses, these bioassays can be applied to numerous monitoring

questions including; (1) time-repeated measurements for variability and concentration patterns (over months, seasons, years), (2) transport through the vadose zone, and (3) partitioning between water, sediment, air at a single location. The ability to address these sorts of issues would greatly enhance our understanding of EDC fate, transport, and impacts allowing for better environmental management.

It is clear that environmental management of EDC contamination in surface and ground water remains a major challenge for the scientific and engineering communities. However, with more research on treatment approaches and technologies, the development of mixing and dilution strategies to maintain EDCs at concentrations below hormonally active levels, and the potential for near real-time field monitoring using biosensors will all provide an excellent set of tools to help address this pressing environmental problem.

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Figure Captions

Figure 1. Summary of EDC bioassays and biosensors including mode of detection, mode of expression, and the specific techniques discussed in the following sections.

Figure 2. Schematic representation of the different processes and compartments that need to be monitored to characterize the fate and transport of EDCs in the environment.

Table 1: Properties of selected EDCs from the literature

EDC	$LogK_{oc}$ (L/Kg)	Solubility (mg/L)	EEF[1.]	CMC[2.] (mg/L)	pK_a
Estradiol	2.55 – 2.80	32.0	$1.0_{a.}$	NA[3.]	10.5 -10.7
17β-Estradiol	3.10 – 4.01	13.0	$1.0_{b.}$	NA	NA
Estrone	2.45 - 3.34	6.0 – 13.0	$0.1 – 1.0_{a.}$ $0.01 – 0.1_{b.}$	none	10.3 - 10.8
Testosterone	3.22	18.0 - 25.0	None[4.]	NA	NA
Nonylphenol (NP)	3.97 – 5.39	4.9 – 7.0	$2.3x10^{-5}– 9.0x10^{-4}{}_{a.}$ $7.2x10^{-7}– 1.9x10^{-2}{}_{b.}$	5 -13	10.28
Nonylphenol ethoxylates (NP1EO-NPnEO)	3.91- 5.64	3.02 – 7.65	$4.0x10^{-6}– 1.3x10^{-5}{}_{b.}$	$4.25x10^{-5}$	NA
Octaphenol	3.54 – 5.18	12.6	$1.0x10^{-5}– 4.9x10^{-4}{}_{b.}$	NA	NA

[1.] Estrogen Equivalent Factor effect relative to estradiol (a.) and relative to 17β-Estradiol (b.) – Ranges include various difference bioassays and estrogen receptors including ER-CALUX, YES, E-Screen transgenic zebrafish, and sheepshead minnows, as well as, both hEH-α and hEH-β receptors.
[2.] Critical Micelle Concentration
[3.] Not available or not found in the literature
[4.] Not an estrogenic EDC

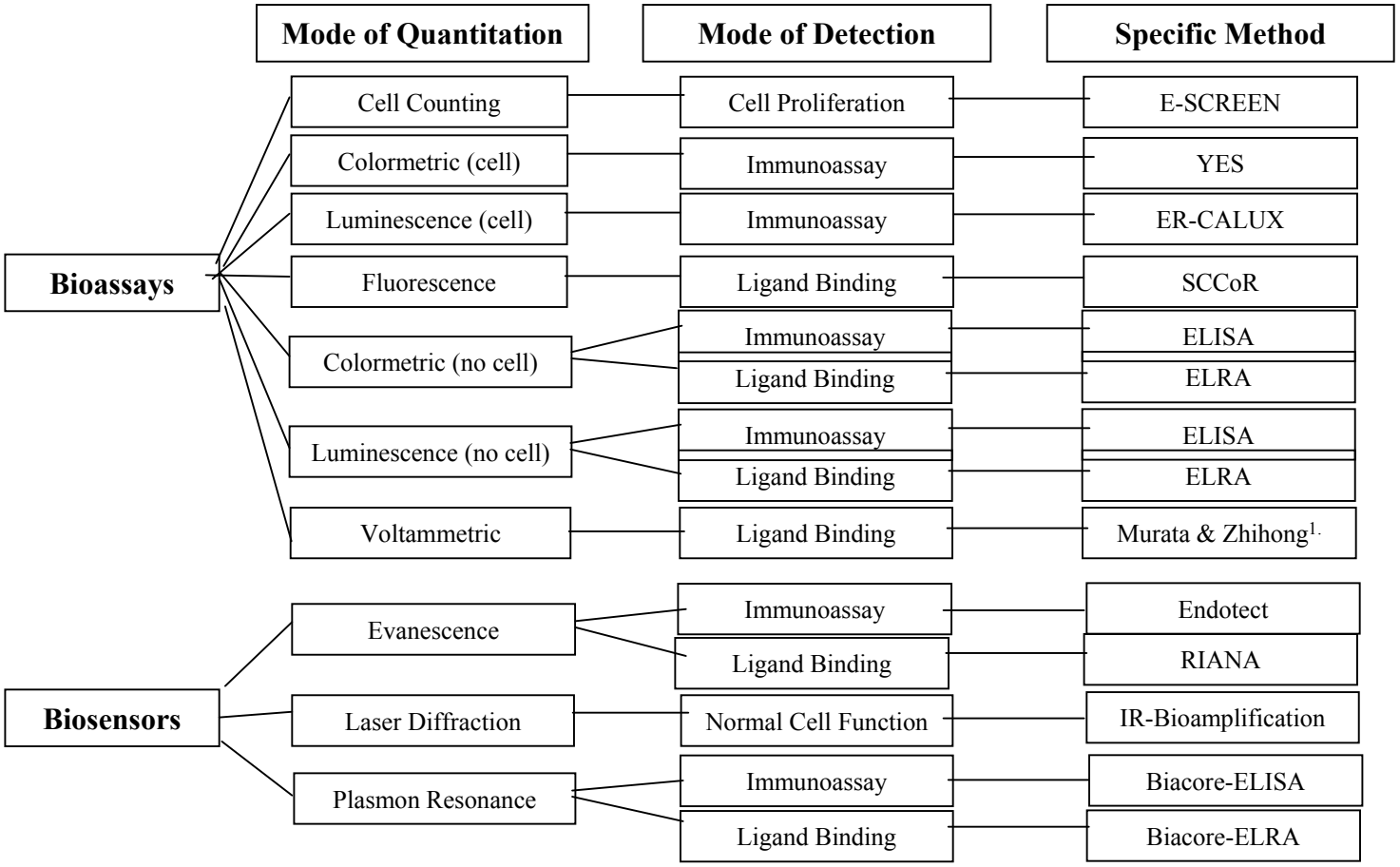
Sources: Petrović et al., (2004); Hanselman et al., (2003); Lee et al., (2003); Folmar et al., (2002); During et al., (2002); Legler et al., (2002a); Ying et al., (2002); Brix et al., (2001); Ferguson et al., (2001); Müller and Schlatter, (1998); Ahel and Giger (1993)

Table 2. Water concentrations up and downstream from a wastewater treatment plant (Sacramento River, near Redding, California, USA)

	Estrone	Estriol	Estradiol
Upstream water, ng/mL	1.4 ± 0.4	1.7 ± 0.2	2.1 ± 0.2
Downstream water, ng/mL	1.5 ± 0.5	2.8 ± 0.7	3.4 ± 0.9
Upstream sediment, ng/g	0.4 ± 0.1	0.5 ± 0.2	0.9 ± 0.3
Downstream sediment, ng/g	0.6 ± 0.2	1.5 ± 0.5	2.1 ± 0.9

Table 3. Water concentrations influent and effluent from Delhi and Hilmar wastewater treatment plants in central California, USA

Estradiol	Hillmar Plant	Delhi Plant
In	4.059	2.607
Out	0.103	0.089
% removal	97.462	96.586
Testosterone		
In	0.480	0.548
Out	0.040	0.040
% removal	91.653	92.748



1. Murata et al. (2001) & Zhihong et al. (1999)

Figure 1

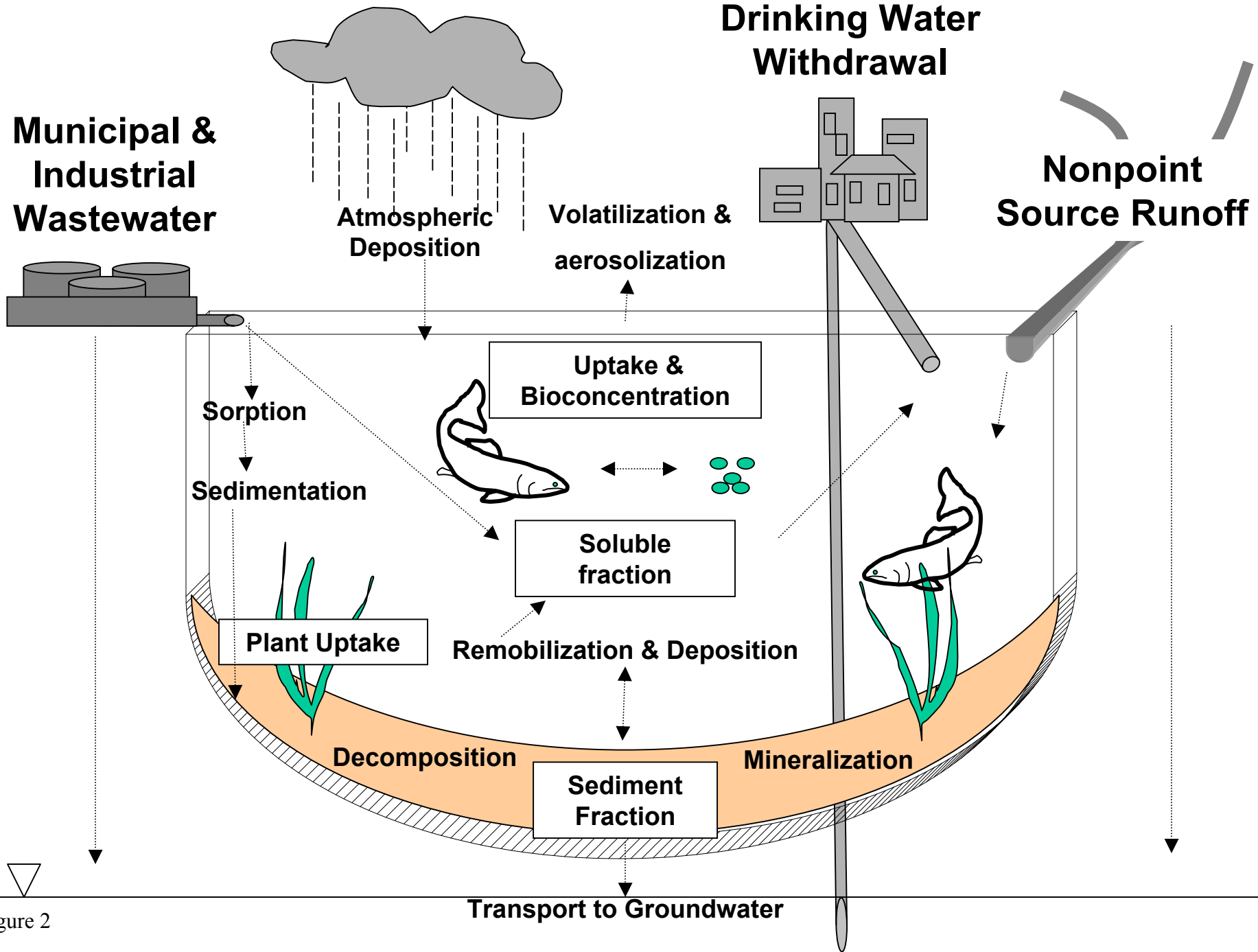

Drinking Water Withdrawal
Municipal & Industrial Wastewater
Nonpoint Source Runoff
Atmospheric Deposition
Volatilization & aerosolization
Uptake & Bioconcentration
Sorption
Sedimentation
Soluble fraction
Plant Uptake
Remobilization & Deposition
Decomposition
Mineralization
Sediment Fraction
Transport to Groundwater

Figure 2