Summary

The purpose of this report is to provide scientific background on the **medaka** bioassay used for the screening of EDs. The results from our project will contribute to the development of fish full life-cycle test (FLC) as well as help the modification of fish early life-stage toxicity test for endocrine disrupting properties. The studies on our project also provide information for toxicity mechanisms of EDs in fish.

In recent years, a global concern has been raised about the potential impact of natural steroid hormones and other chemicals mimicking the effects of hormones in aquatic ecosystems, particularly because of the possibility of adverse effects on sexual differentiation and reproduction in wildlife. However, none of the existing methods for *in vivo* test were sufficient for screening and testing for the endocrine disruption of these substances. Therefore, we commenced, in 1998, a series of studies to develop suitable test methods with fish.

Initially, it was important to establish what kind of biological effects was observed in response to exposure to EDs as well as to determine the changes in sensitivity during various stages of the fish life-cycle. Further, we had to identify what kind of chemicals had biological effects of this nature. FLC is a test method covering all stage of life cycle. Then, we confirmed usefulness of **medaka** as a test model in FLC using reference chemicals.

In the early stage of our project, frameworks of several test methods were developed. The fish partial life stage toxicity test (PLC) is a test focused on a sexually critical developmental-stage. A test for a steroidgenically active stage was termed the reproduction test (REP). In addition, a very sensitive to biological effects and more cost-effective screening method has been developed. Vitellogenin (VTG) assay is a test to detect vitellogenin induction in liver as an effect of EDs. The new **medaka** strains, such as FLF and d-rR, in which sex can be distinguished in a simple manner as observing leucophores or body color have been used for the development of these tests.

Medaka is an ideal bioassay model for research and testing in toxicology. It has been widely used for studies on the effects of radiation, carcinogens and chemicals and has been recommended as a test species in many existing standard eco-toxicological guidelines, such as OECD test guidelines. Furthermore, **medaka** is an attractive model organism for evaluating life-cycle toxicity in both parental and progeny generations because of its short maturation time-within six to eight weeks after hatching. This species can spawn daily under summer photoperiod, temperature conditions with proper food regime. The first chapter on this report describes suitability of medaka as test organ to detect the effect of EDs.

In vitro studies also have been developed in order to screen many of the chemicals of concern. To date, **medaka** estrogen receptor (*me*ERá and *me*ERâ) binding assay, *me*ERá and *me*ERâ reporter gene assay and **medaka** androgen

receptor (*me*AR) reporter gene assay have been developed. The second chapter describes the reporter binding assay and receptor gene assay.

The current testing strategy by the Ministry of the Environment, Japan, requires evaluating the estrogenic or androgenic activity of chemicals (see attached Testing Scheme). In screening, VTG assay is conducted to detect estrogenic activity of suspected EDs. Then, PLC is performed to elucidate their effects on the sexual differentiation of **medaka,** because this test had higher sensitivity for several estrogens than the REP that sexually matured and constantly spawning fish are exposed to chemicals during the reproductive phase.

In the PLC, fish are exposed to chemicals from an embryo stage to almost matured adult stage. In the short term PLC, fish are exposed to chemicals during embryo stage to early-matured adult stage. The parameters investigated in the tests are hatching success (including next generation), survival, growth, reproduction, gonado-somatic index (GSI), hepato-somatic index (HSI), secondary sex characteristic (sec. SEX), gonadal histology, VTG concentration in liver.

If adverse effects were not observed in the PLC, the FLC is not necessary. If adverse effects were observed in the PLC, FLC would then be conducted.

As a result of our activities to date, the VTG assay had been conducted with 20 chemicals*[1] and the REP has been performed with nonylphenol and bisphenol A. The PLC was done with 12 chemicals*[2] and FLC was performed with 4 chemicals*[3] respectively. Some of OECD reference chemicals such as 17â - estradiol, ethynylestradiol and methyl testosterone were tested. We also prepared protocols for the PLC and FLC that were submitted to the OECD secretariat in December, 2002 (Appendix ‡T-‡Tand ‡U).

An important study had been developed on the mechanism of sexual differentiation in **medaka** by information from basic and scientific data. In order to make clear the effects of EDs on the mechanism of sexual differentiation **medaka**, some studies have been conducted with utilizing genetic technology as follows. (1) Cloning a series of genes associated with the regulation of sex differentiation in **medaka**. (2) Preparing of a gene, which appears in a period of differentiation of sex. (3) Development of the DNA chip containing a series of gene associated with the sexual differentiation. A series of

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[1] Tributyltin, 4-octylphenol, nonylphenol, di-n-butylphtalate, octachlorostyrene, benzophenone, di-cyclohexylphtalate, di-(2-ethylhexyl)phtalate, butylbenzylphtalate, di-ethylphthalate, di-(2-ethylhexyl)-adipate and triphenyltin are selected in 2000. Pentachlorophenol, amitrol, bisphenol A, 2,4-dichlorophenol, 4-nitrotoluene, di-pentylphtalate, di-hexylphtalate and di-propylphtalare are selected in 2001.

[2] Tributyltin, 4-octylphenol, nonylphenol, di-n-butylphtalate, octachlorostyrene, benzophenone, di-cyclohexylphtalate, di-(2-ethylhexyl)phtalate, butylbenzylphtalate, di-ethylphtalate, di-(2-ethylhexyl)-adipate and triphenyltin

[3] Tributyltin, 4-octylphenol, nonylphenol and di-n-butylphtalate

genes associated with regulating the sexual differentiation have been cloned and prepared to develop a DNA macro array. The sex-determination gene of **medaka**, DMY (Y-specific DM-domain gene) has been first found in non-mammalian vertebrates. The results of these studies with utilizing the gene technology will be useful to make clear the effect of EDs on the mechanism of the sexual differentiation in **medaka**. The last chapter describes genetic approaches to understanding the basic mechanisms of DES actions on sexual differentiation in medaka.

Testing Scheme in Evaluation of the Endocrine Disrupting Activities in Fish