



The 11th UK-Japan Annual Scientific Workshop on Research into Environmental Endocrine Disrupting Chemicals



**Osaka, Japan
12-14th November 2009**



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into Environmental Endocrine Disrupting Chemicals



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Abstracts and Programme Information



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The 11th UK-Japan Annual Scientific Workshop on Research into Environmental
Endocrine Disrupting Chemicals
RIHGA Royal Hotel Osaka, Japan 12-14th November 2009

Programme

Thursday, 12th November

2F "Tsuta Room"

19:00 - 21:00 **Welcome Reception**

Opening Address/Welcome by

Teruyoshi Hayamizu (Ministry of the Environment)

Ian Dalton (Department for Environment, Food and Rural Affairs
(DEFRA))

Friday, 13th November

Tower Wing 28F "Emerald Room"

WELCOME and INTRODUCTION

9:00 - 9:30 **Welcome to the Science**

Opening Remarks

Mike Roberts (Department for Environment, Food and Rural Affairs
(DEFRA))

Logistics, meeting events and an overview of UK-J 2004-2009

Taisen Iguchi (National Institutes of Natural Sciences)

SESSIONS

CORE PROJECT 1

Chairs: David Sheahan (Centre for Environment, Fisheries & Aquaculture Science
(CEFAS))

Norihisa Tatarazako (National Institute for Environmental Studies)

9:30 - 10:10 **Investigations into reduction of oestrogenic activity in
wastewater and evaluation of biological significance (C1)**

Monika Jürgens (Centre for Ecology and Hydrology (CEH))

Hiroaki Tanaka (Kyoto University)

Mizuhiko Minamiyama (Public Works Research Institute)



CORE PROJECT 2

Chairs: David Sheahan (Centre for Environment, Fisheries & Aquaculture Science (CEFAS))

Norihisa Tatarazako (National Institute for Environmental Studies)

- 10:10 - 11:00 **Evaluation of the endocrine disrupting potency of chemicals and other pollutants using the stickleback model**
- Optimization of *in vivo* exposure condition to detect androgenic potency of chemicals using the three-spined stickleback (C2.1)
Masaki Nagae (Nagasaki University)
 - Two exposure scenarios to a mixture of endocrine disrupting chemicals using the three-spined stickleback (C2.2)
Ioanna Katsiadaki (Centre for Environment, Fisheries & Aquaculture Science (CEFAS))
 - Exposure to silver nanoparticles alters gene expression in the brain of the three-spined stickleback (*Gasterosteus aculeatus*) (C2.3)
Tim Bean (Centre for Environment, Fisheries & Aquaculture Science (CEFAS))
- 11:00 - 11:20 **Coffee Break**

CORE PROJECT 3

Chairs: Christina Lye (Enviresearch Ltd.)

Yasuhiko Ohta (Tottori University)

- 11:20 - 12:00 **Investigating mechanisms of feminisation and oestrogenic responses in fish (C3)**
Anke Lange (University of Exeter)
Shinichi Miyagawa (National Institutes of Natural Sciences)
- 12:00 - 13:30 **Lunch and Poster Presentations**



Saturday, 14th November

Tower Wing 28F “Sapphire Room”

INVITED PRESENTATIONS

Chairs: Charles Tyler (University of Exeter)

Taisen Iguchi (National Institutes of Natural Sciences)

- 8:40 - 9:10 **From Modes of Action to Managing Risks: an Update on Screening and Testing for Endocrine Disruptors** **(S1)**
Tom Hutchinson (Centre for Environment, Fisheries & Aquaculture Science (CEFAS))
- 9:10 - 9:40 **Chemical Selection and Evaluation for Risk Assessment - A UK and European Perspective** **(S2)**
Mike Roberts (Department for Environment, Food and Rural Affairs (DEFRA))
- 9:40 - 10:10 **Activities on Testing and Assessment of Endocrine Disruptors by MOE and Chemical Management Policy in Japan** **(S3)**
Teruyoshi Hayamizu (Ministry of the Environment)

SHAPING NEW CORE PROJECTS AND SUMMING UP

Chairs: Charles Tyler (University of Exeter)

Taisen Iguchi (National Institutes of Natural Sciences)

- 10:10 - 10:40 **Key Objectives for members in the new core activity framework in 2010.**
- 10:40 - 11:00 **Discussion**
- 11:00 - 11:30 **Coffee Break**
- 11:30 - 11:50 **Summing Up**
Charles Tyler (University of Exeter)
Taisen Iguchi (National Institutes of Natural Sciences)
- 11:50 - 12:00 **Closing remarks**
Ian Dalton (Department for Environment, Food and Rural Affairs (DEFRA))
Teruyoshi Hayamizu (Ministry of the Environment)



12:00 - 12:15 **Coffee Break**

SIGNING CEREMONY FOR EXTENSION OF UK-JAPAN AGREEMENT

12:15 - 12:30 Japan: Teruyoshi Hayamizu (Ministry of the Environment)

UK: Ian Dalton (Department for Environment, Food and Rural Affairs
(DEFRA))

Sunday, 15th November

8:00 - 19:00 Excursion- Study visit to Otsu and Kyoto



Talk Abstracts

CORE PROJECTS

- (C1) Investigations into Reduction of Oestrogenic Activity in Wastewater and Evaluation of Biological Significance
Hiroaki Tanaka, Mizuhiko Minamiyama, Yutaka Suzuki and Andrew Johnson
- (C2.1) Optimization of *in vivo* Exposure Condition to Detect Androgenic Potency of Chemicals Using the Three-spined Stickleback
Masaki Nagae, Subaru Matsuo, Tsuyoshi Iwata, Masanobu Sakai, Yoshinao Katsu, Nobuyuki Ohkubo, Matsubara Takahiro, Kiyoshi Soyano, Alexander P. Scott and Ioanna Katsiadaki
- (C2.2) Two Exposure Scenarios to a Mixture of Endocrine Disrupting Chemicals Using the Three-spined Stickleback
Ioanna Katsiadaki, Matthew B. Sanders, Yvonne Allen, Grant D. Stentiford and Alexander P. Scott.
- (C2.3) Exposure to Silver Nanoparticles Alters Gene Expression in the Brain of the Three-spined Stickleback (*Gasterosteus aculeatus*)
Bean Tim P., Sanders Matthew B., Lyons, Brett, Christian Paul, Williams Tim D., Katsiadaki Ioanna and J. Kevin Chipman
- (C3) Investigating Mechanisms of Feminisation and Oestrogenic Responses in Fish
Anke Lange, Yoshinao Katsu, Shinichi Miyagawa, Taisen Iguchi and Charles R. Tyler
- (C4) Development of Methods for Detecting and Assessing Impacts of Chemicals on Amphibians
Minoru Takase and Daniel Pickford

INVITED PRESENTATIONS

- (S1) From Modes of Action to Managing Risks: an Update on Screening and Testing for Endocrine Disrupters
Tom Hutchinson
- (S2) Chemical Selection and Evaluation for Risk Assessment - A UK and European Perspective
Mike Roberts
- (S3) Activities on Testing and Assessment of Endocrine Disruptors by MOE and Chemical Management Policy in Japan
Teruyoshi Hayamizu



(C1) Investigations into Reduction of Oestrogenic Activity in Wastewater and Evaluation of Biological Significance

Hiroaki Tanaka^{1*}, Mizuhiko Minamiyama², Yutaka Suzuki² and Andrew Johnson³

¹Kyoto University, ²Public Works Research Institute and ³Centre for Ecology and Hydrology

Studies carried out

(1) Developing improved analytical methods for estrogens and their conjugates

Methods were developed based on amino-propyl SPE and LC/MS/MS with high recoveries of free and conjugated estrogens could allow us to understand their fates in sewage treatment and receiving waters. The detection limits of this method ranged from 0.2 to 0.8 ng/L for studied free and conjugated estrogens. A GC-HRMS based analytical procedure for EE2 in domestic wastewater was also developed. The procedure demonstrated in this study is innovative in terms of levels of detection of the EE2. The detection limit of this method was 0.05 ng/L in the original sample.

(2) Assessing the fate of estrogens and their conjugates in the environment using laboratory simulations

Biodegradation batch tests of estrogens with untreated sewage, activated sludge and river water in Japan were conducted. The values of the degradation rates, k_s , were glucuronide conjugates \gg E2 $>$ E1 \gg sulfate conjugates in both the sewage and river water samples. Degradation rates for all compounds in the river water samples were much smaller than those of the sewage samples.

(3) Assessing the performance of tertiary treatments to remove estrogens

The results of field surveys in Japan and the UK demonstrated that 1 to 6 mg/L of ozone dose after biological treatment process could significantly decrease free estrogens to below detection limit. This indicates ozonation following biological treatment is a prospective and stable method for reducing estrogen concentrations in addition to improvement of biological treatment.

(4) Developing novel sewage treatment approaches to assist estrogen removal

The tertiary process contained plastic media for the attachment of microorganisms which can degrade estrogens, and the media were fluidized by aeration. Hydraulic retention time was set at 2 hours. The process could effectively remove estrogens of around 20ng/L in the STP effluent to below the detection limit. A relationship was observed between the estrogen removal rate and the TOC concentration in the STP effluent, which suggests the co metabolism of estrogens with organic matter contained in the STP effluent.

(5) Reviewing and comparing the performance of UK and Japanese sewage treatment plants in removing estrogens

The occurrence of estrogens in sewage, treated sewage and receiving river water between the UK and Japan was examined using identical analytical methods. The largest difference between the



countries was that ethynylestradiol (EE2) was prevalent in sewage effluent in UK while it was below detection limit in all samples bar one in Japan. In both the countries glucuronide conjugates of estrogens exist in sewage, but are transformed before reaching the effluent in most cases. In contrast a significant proportion of the sulfate conjugates pass through into the effluent. For activated sludge 17 β -estradiol (E2) was more effectively reduced in Japan, while estrone (E1) was more effectively reduced in the UK. DO concentration was found to be a very important operation factor for the estrogen removal in a STP with relatively short solid retention time (SRT).

(6) Field monitoring studies to review discharge and fate of estrogens in UK and British rivers

Five field surveys were conducted in Yodo River systems to understand the fate of estrogens in the waters receiving STP discharge. The results indicated that most of estrogen load is derived from STP discharge in this river basin and mainly consists of E1. Superficial degradation rates of Katsura River, Uji River and Yodo Rivers estimated from the observed fluxes fluctuated to large extent based on the seasons. The results also suggested sorption photodegradation may play an important role in removal in the real environment.

(7) National comparisons of flow regimes and dilutions to assess relative estrogen exposure for UK and Japan

UK and Japan are similar in being highly developed densely populated islands. Thus, the amount of estrogen discharge and endocrine disruption might also have been expected to be similar. But whilst the estrogen equivalents discharge was calculated to be quite similar, Japan has at least three-fold more available annual dilution suggesting an overall smaller impact than England might be expected.

(8) Focused catchment comparisons of flow regimes and dilutions to assess relative estrogen exposure for UK and Japan

In an effort to obtain a more representative comparison of potential endocrine disruption risks between England and Japan, detailed population and hydrological studies were carried out on 31 Japanese and English catchments spread geographically around the two nations. Rather than provide one estimate of risk based on mean flow, 25thile, mean and 75thile flow values were used to capture a spread of potential estrogen concentrations. This approach allows for the dynamics of different hydrological systems to be taken into account. The estimated E2-equivalents for the Japanese catchments was similar to actual measurements except for seven examples.

(9) Perspectives

The methods and approaches used in the study of estrogens in the CORE 1 study group are very suitable to adapt to the assessment of other emerging threats to the freshwater ecosystem. Projects are already ongoing to look at a range of pharmaceuticals, personal care products and nanoparticles in the sewage and receiving water environments of UK and Japan.



(C2.1) Optimization of *in vivo* Exposure Condition to Detect Androgenic Potency of Chemicals Using the Three-spined Stickleback

Masaki Nagae^{1*}, Subaru Matsuo¹, Tsuyoshi Iwata¹, Masanobu Sakai¹, Yoshinao Katsu², Nobuyuki Ohkubo³, Matsubara Takahiro³, Kiyoshi Soyano⁴, Alexander P. Scott⁵ and Ioanna Katsiadaki⁵

¹Faculty of Environmental Studies, Nagasaki University, Japan, ²Graduate School of Life Science, Hokkaido University, Japan, ³Hokkaido National Fisheries Research Institute, Fisheries Research Agency, Japan, ⁴Institute for East China Sea Research, Nagasaki University, Japan, and ⁵Cefas Weymouth Laboratory, UK

The use of specific biomarker enables quick and precise evaluation of the hormonal potency of environmental chemicals. Male three-spined stickleback (*Gasterosteus aculeatus*) produces a glue protein, spiggin, which is used in the building of the nest that females enter in order to spawning. The synthesis of spiggin is strongly controlled by androgen. However, there is room for basic and comprehensive understandings of spiggin synthesis in kidney. In this study, the effect of *in vivo* exposure condition (water temperature, photoperiod and duration) on spiggin synthesis was examined.

Immature male and female sticklebacks were treated with 17 α -methyltestosterone (MT) at various exposure conditions. Kidney RNA samples were extracted and applied to spiggin mRNA quantification.

Water temperature strongly influenced spiggin synthesis in MT treated stickleback. At 15 degrees centigrade, spiggin mRNA levels in kidney were extremely high, there was closely positive correlation between MT concentration and spiggin synthesis in kidney. On the other hand, at lower temperature, particular in 5 degrees centigrade, spiggin mRNA levels markedly decreased. The condition of photoperiod had no marked influence on the spiggin synthesis in kidney. At 0.01 mg/L MT exposure (NOEL of MT on spiggin synthesis during 7days in our exposure system), the extension of exposure duration over 7days was no effect on spiggin synthesis. From these results, the optimal exposure condition to detect spiggin mRNA was proposed (7days at 15 degrees centigrade). The results during this 5 years also will be talked and summarized in this presentation.



(C2.2) Two Exposure Scenarios to a Mixture of Endocrine Disrupting Chemicals Using the Three-spined Stickleback

Ioanna Katsiadaki^{*}, Matthew B. Sanders, Yvonne Allen, Grant D. Stentiford and Alexander P. Scott.

Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK

The use of the stickleback as a model species in ecotoxicology and in particular chemical endocrine disruption is increasing. The stickleback offers two unique tools for the study of endocrine disrupting chemicals (EDCs), the androgen-regulated protein spiggin and the well-documented reproductive behaviour. In addition it is one of the few recognised species for testing that inhabits marine environments.

Here we describe histopathological and biomarker (vitellogenin, spiggin) responses in adult fish that were exposed over a six-month period to a model oestrogen and a model antiandrogen alone and in combination. The experiment was conducted twice. The design between the two exposures was similar but the first followed an episodic/pulse exposure whilst the second followed the more usual constant concentration exposure. During the episodic exposure the fish were initially treated with a high concentration of EDCs, which was subsequently reduced to below measured environmental levels, whilst all the concentrations used in the constant exposures were environmentally relevant and stable over time.

The responses between the two exposure scenarios were dramatically different both in terms of biomarker data and gonadal histopathology. Severe intersex fish were observed in the episodic mode of exposure but not in the constant mode. In addition there were signs of synergism between the two model chemicals used; fish treated with the binary mixture were more feminised than fish treated with the oestrogen alone. This chemical synergism was also reflected in the VTG titres regardless of the mode of exposure. The findings are discussed in terms of risk assessment of endocrine active compounds in the aquatic environment with particular emphasis on the high antiandrogenic activity documented in certain body waters.



(C2.3) Exposure to Silver Nanoparticles Alters Gene Expression in the Brain of the Three-spined Stickleback (*Gasterosteus aculeatus*)

Bean Tim P.^{1*}, Sanders Matthew B.¹, Lyons, Brett¹, Christian Paul², Williams Tim D.³ and Katsiadaki Ioanna¹ and J. Kevin Chipman³

¹Cefas Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK, ²School of Chemistry, The University of Manchester, Oxford Road, Manchester, M13 9PL, UK and ³School of Biosciences, The University of Birmingham, Birmingham, B15 2TT, UK

Increased utilization of nanomaterial within industrial and remediation processes is likely to increase environmental exposure to engineered nanoparticles (ENPs). Several studies have reported biological effects following exposure to ENPs such as carbon nanotubes, metal oxides and Quantum Dots (QDs). However, much of this work has focused on air-born exposure, and there is little understanding of the effects on the aquatic environment. Recently the use of nanosilver in industrial processes and domestic products has raised regulatory and environmental concerns over the paucity of knowledge regarding the risks posed by these ENPs. Using a stickleback cDNA microarray, changes in transcript levels in brain and liver tissue of fish exposed for 48 hours to silver QDs ($\phi=13\pm 7\text{nm}$) coated with methyl polyethylene glycol (mpeg) at doses of $5\text{-}500\mu\text{gL}^{-1}$ or bulk silver ($\phi >500\text{nm}$) at $500\mu\text{gL}^{-1}$ were compared with fish exposed to mpeg ($500\mu\text{gL}^{-1}$) alone. Analysis of the data indicates that a cohort of genes displayed dose-dependent expression in brain but not liver tissue following exposure to silver nanoparticles, and that the responses in brain to $500\mu\text{gL}^{-1}$ silver QDs were broadly similar to those elicited by exposure to bulk silver. Results from this study are being used to develop specific gene response markers for studies assessing the risks posed by silver nanoparticles to the aquatic environment.



(C3) Investigating Mechanisms of Feminisation and Oestrogenic Responses in Fish

Anke Lange^{1*}, Yoshinao Katsu^{2,3}, Shinichi Miyagawa², Taisen Iguchi² and Charles R. Tyler¹

¹School of Biosciences, University of Exeter, United Kingdom, ²Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Japan and ³Graduate School of Life Science, Hokkaido University, Japan

A variety of natural and man-made chemicals can mimic hormonal activities and bind to hormone receptors, resulting in the modulation of biological processes. Aquatic species are especially vulnerable to exposure to environmental endocrine disrupting chemicals as these exposures can be both considerable and continuous. Hormonally active chemicals have been shown to not only cause disruption of sexual differentiation and development and to impact on reproduction in various fish species, but to also have wider health effects. Oestrogens and oestrogen-mimicking compounds that act via oestrogen receptors are known to play a major role in the disruption of sexual differentiation and development of wild fish. Some of the most potent environmental oestrogens include natural sex steroids and pharmaceutical oestrogens used in contraceptives and hormone replacement therapies.

In our collaborative research under the UK-Japan partnership we have undertaken three main themes of research to develop a greater understanding on the health effects of exposure to environmental oestrogens, namely:

- a) the development and application of molecular tools in a range of fish species, with a particular focus on the native species roach (*Rutilus rutilus*) and medaka (*Oryzias latipes*)
- b) short- and long-term *in vivo* exposures of roach to environmental concentrations of oestrogens or sewage effluent to investigate their effects on sexual differentiation and development and their impact on reproduction in exposures to effluents
- c) studies to investigate the comparative sensitivities of a range of different fish species to natural and synthetic steroidal and environmental oestrogens, conducted through both *in vivo* and *in vitro* studies.

Given the lack of genomic resources for the roach at the beginning of this partnership, we cloned a suite of 42 genes involved with sexual development and function in the roach and developed q RT-PCR assays for quantifying their expression. These assays were then used to study the responsiveness of sub-adults to oestrogen during early life, to assess responsiveness to oestrogen re-challenge in later life after an initial challenge in early life (to investigate for sensitisation) and to probe the molecular mechanism of feminisation in roach. Further molecular studies have included the isolation of a gene that might be a potential biomarker for testis-ova induction (in the medaka) and initial investigations have been made into the presence of this gene in roach.

Continuing on the molecular theme, our Japanese partners have cloned oestrogen receptors from the roach, medaka and seven other fish species, that have been applied in the development of reporter gene assays to investigate their interactions with steroidal oestrogens and oestrogen mimics *in vitro* and to analyse the ligand- and species-specificity of fish ERs. This work has been extended to assess



the transcriptional activities of fish ER α s exposed to DDT, its metabolites and environmental chemicals. Overall, we have shown that the specificity of fish ER α transcriptional activity to oestrogenic compounds differs between species. In addition, we have compared the ligand-specificity of three zebrafish ERs and found that each ER has its own oestrogen-specificity. Furthermore, we have also compared the ligand-dependent induction of zebrafish ERs using the environmental oestrogen mimics and intriguingly, zebrafish ER β 1 was more sensitive to all environmental chemicals examined compared to ER α and ER β 2, suggesting that ER β 1 may be the main target receptor for oestrogens and other environmental oestrogenic chemicals.

Our *in vitro* work has been complimented with a series of *in vivo* studies. In studies on roach we have exposed fish to both 17 α -ethinyloestradiol (EE₂, including at environmentally relevant concentrations), and to WWTW effluents for periods up to 3 years and induced all the feminised phenotypes we see in roach in UK effluent-contaminated rivers, including intersex and even complete gonadal feminisation. In further *in vivo* exposures to dihydroequilenin (a horse oestrogen used in hormone replacement therapy) for a period of 21 days we have shown that rainbow trout (*Oncorhynchus mykiss*) are more sensitive to this oestrogen compared with common carp (*Cyprinus carpio*) (LOEC for vitellogenin induction of 0.6 ng/L versus 420 ng/L, respectively). Similarly, for a study exposing rainbow trout and three-spined stickleback (*Gasterosteus aculeatus*) in the same tanks for 7 days to EE₂, VTG induction occurred at the lowest concentration tested in trout (4 ng/L), but no induction was evident in sticklebacks, even at 8 ng EE₂/L. Recently, we completed an exposure in which four temperate species (roach, carp, stickleback and rainbow trout) were exposed in the same tanks to two concentrations (2 and 10 ng/L) of EE₂. After 7 days, hepatic *vtg* gene expression was only significantly induced in rainbow trout and male roach exposed to 10 ng/L, but not in carp, stickleback or female roach. All these findings strongly support the *in vitro* data suggesting considerable species differences in the sensitivity of fish to oestrogens, but studies *in vivo* need to consider factors such as sex or developmental stage and the analyses are thus more complex. A second multi-species exposure will be carried out for warm water species (medaka, zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*)) using the same design as for the coldwater species described above) to establish their comparative sensitivity and responsiveness to EE₂ and for comparison with the *in vitro* ER receptor binding/activation data. We now propose to examine the expression level and tissue distribution of the three ERs in detail in selected fish species to help unravel the functional significance of these combined *in vitro* and *in vivo* findings.

A final of work that we have conducted under this initiative is to start to investigate the reproductive capabilities of roach that have been exposed to WWTW effluent throughout their lives (3 years). In this experiment effluent exposed fish have been placed with fish maintained in clean water and the breeding success of individuals is being determined by parentage analysis on the offspring using DNA microsatellites.

In the UK-J partnership scheme (2005-2010) this collaboration has been extremely successful and rewarding with 9 joint publications and 5 research exchanges between the partner laboratories.



(C4) Development of Methods for Detecting and Assessing Impacts of Chemicals on Amphibians

Minoru Takase^{1*} and Daniel Pickford²

¹Hiroshima University and ²Brunel University

The aim of Core 4 study in the UK-J project during the past five years is to develop and validate a suite of methods for assessing the impacts of environmental contaminants on amphibians, and was consisted of three major objects such as development of environmental assay tools, development of biological test method, and model test.

In the early years, the Core 4 project focused on wild amphibians. UK side provided Japan side a lot of hisotorical data of amphibian population and agrochemicals accumulated in UK as data of the DEFRA-founded project. Count data of live toads (crossing) and road mortalities from 'Toads on Roads' schemes over the last 20 years provide temporal count data as estimates of local abundance of toads. Without adjustment these count data provide crude estimates of population trajectories at best. Data from the Environment Agency of England and Wales' POPPIE database provide an indication of potential exposure of wildlife to agrochemicals. On this basis 10 sites were selected in England for environmental sampling using Passive Accumulation Devices (PADs: SPMD and POCIS) to sequester water-borne contaminants. Extracts were generated at nominal 20,000x concentration and initially screened in Yeast Recombinant Assays (YES and YAS) for estrogenic, anti-estrogenic, androgenic and antiandrogenic activity. PADs offer many advantages for environmental sampling in support of a population level assessment of impact of chemical contaminants. It is hoped that among other areas, use UK-J cooperation on use of PADs for environmental sampling, (cross-)validation of relevant and informative bioassays, and development of higher resolution spatial analysis models using GIS methodologies will benefit our collective efforts to improve assessments of the impact of chemical contaminants on amphibians. Japan side explored common method for amphibian survey and common frame of amphibian ecological information between UK and Japan, on the basis of the information from UK side and the related-literatures.

Concerning development of biological assay tools, isolation of cDNAs encoding steroid hormone receptors such as ERs and TRs, development of reporter gene assay systems using ERs and TRs, isolation of estrogen-responsive genes, and production of sex-reversed male frog have been done in the core 4 project. In these studies, the West African clawed frog, *Xenopus (Silurana) tropicalis* has been used as standard experimental amphibian, because of its diploid genome and shorter generation. These advantages and the increasing availability of genomic information for this species also lend it considerable potential as an alternative to the South African clawed frog (*X. laevis*) for ecotoxicological studies, in particular in relation to endocrine disruption. These tools will support in lab assay system using *X. (S.) tropicalis*. Furthermore, antibody for Vtg purified from one species of wild Japanese amphibian, *Rana japonica*, which looks like *R. temporaria* living in UK, was prepared, and one assay system for wild amphibian Vtg using the antibody was constructed.

Creating a standard database on development of the *X. (S.) tropicalis* tadpole has been identified



as a key objective of Project 4 of the UK-Japan Research Liason on Endocrine Disrupter Research. Studies are being undertaken in Japan and UK to document comprehensively the effects of the model estrogen 17 α -ethynylestradiol (EE2) on larval development and sexual differentiation of *X. (S.) tropicalis*. At each sampling point during development of the tadpole, individual number, day, developmental stage, whole body length, snout-vent length, hind limb length, wet weight, and area of thyroid gland were measured and recorded and larvae were fixed for histological analysis. In addition, gene expression in developing gonads was analyzed comprehensively using microarray technique. Data from both Japanese and UK studies on effects of the model estrogen EE2 on *S. tropicalis* are consistent with an earlier report on effects of EE2 on sex differentiation in *X. (S.) tropicalis* tadpole by Pettersson *et al.* (2006) and will contribute to the growing body of knowledge that supports more widespread use of this species as vertebrate model. Histological features of gonad and thyroid gland in larval and metamorph *X. (S.) tropicalis* offer considerable utility as biomarkers for assessing and evaluating impacts of estrogenic and thyroid active chemicals in amphibians for hazard characterization in chemical risk assessment programs. Further research undertaken as part of this project, will include measurement of serum vitellogenin concentrations during development of the *X. (S.)* tadpole exposed to EE2, and assessment of reproductive endpoints in adult *X. (S.) tropicalis* exposed to EE2 during larval development. These complementary approaches will further contribute to the creation of a standard database for *X. (S.) tropicalis*.



(S1) From Modes of Action to Managing Risks: an Update on Screening and Testing for Endocrine Disrupters

Tom Hutchinson

Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, UK

Reflecting the significant global concern over endocrine disrupting chemicals (EDCs) and the health of people and wildlife, the international investment in research into screening and testing methods has significantly advanced in recent years. As an example, many scientists in OECD-member countries have contributed to the development of important test guidelines for EDC screening in mammals, fish and amphibians. Test guidelines of longer duration (eg partial and full lifecycle tests) are currently being developed for several groups of organisms to support decision making in risk assessments.

Recognising the complexity of risk assessment scenarios for different chemicals, the OECD has also developed a valuable conceptual framework comprising five flexible levels of information: (Level 1) sorting and prioritisation based on existing information; (Level 2) *in vitro* assays providing mechanistic data; (Level 3) *in vivo* assays providing data about single endocrine mechanisms and effects; (Level 4) *in vivo* assays providing data about multiple endocrine mechanisms and effects; and (Level 5) *in vivo* assays providing data on effects from endocrine and other mechanisms.

Case studies of EDCs will be presented in the context of the OECD conceptual framework, with a focus on the mode-of-action of the selected chemicals and how this can be used to guide testing needs and an efficient risk assessment approach. The importance of understanding the quartet of key protein targets in toxicology will be emphasised (namely, enzymes, ion channel blockers, receptors and transporter proteins). This reflects the wider mode-of-action approach now being actively promoted in Europe as part of the Targeted Testing Strategy (also known as ‘Intelligent Testing Strategy’) for agrochemicals, industrial chemicals (under REACH) and pharmaceuticals. Implications for mixtures testing and cumulative risk assessment will also be discussed in the context of the significant scientific achievements made through the highly valued UK-Japan Partnership.



(S2) Chemical Selection and Evaluation for Risk Assessment - A UK and European Perspective

Mike Roberts

Chemicals and Nanotechnology Division, Department for Environment, Food and Rural Affairs (Defra), UK

Like other EU Member States, the UK takes the view that chemicals considered as posing potential human and environmental risks should be proposed for full risk assessment at the European, rather than the national, level. Thus any necessary controls which emerge are agreed across the European Union.

The EU regulatory framework for chemicals, and how chemicals are selected for assessment, has changed in recent years. This paper summarises these changes with respect to the REACH (**R**egistration, **E**valuation, **A**uthorisation and **R**estriction of **C**hemicals) Regulation and the Regulation of Plant Protection Products. Some future trends, such as assessment of mixtures of chemicals and test method developments, are discussed, to draw a focus on the implications for the UK-Japan Partnership.



(S3) Activities on Testing and Assessment of Endocrine Disruptors by MOE and Chemical Management Policy in Japan

Teruyoshi Hayamizu

Environmental Health and Safety Division, Environmental Health Department, Ministry of the Environment, JAPAN

Since the 1960s, it has been a matter of concern, from the global observation of wildlife, that some chemicals in the environment may behave similarly to endogenous hormones and disrupt their endocrine systems. In 1996, a group of Dr. Theo Colborn, an American zoologist, published “Our Stolen Future” pointing out the serious effects of chemicals on wildlife, and sensationally arose the fears that there may have similar effects on humans as well.

In May 1998, Ministry of the Environment (MOE) summarized its basic policy on endocrine disruptors in “the Strategic Programs on Environmental Endocrine Disruptors -SPEED '98-” based on the discussions by the expert panel. According to the programs, MOE promoted various measures including monitoring of chemicals suspected to have endocrine disrupting effects in the environment, pursuing a variety of possibly occurring effects in the organisms via endocrine system, and participating in international collaborative studies.

MOE reflected on the fact that while research conducted under the SPEED '98 delivered a certain level of progress in understanding endocrine disruption of chemicals, of which we knew only a little at the time, a misunderstanding arose in the public, giving the impression that the substances listed for priority investigation were identified as endocrine disruptors.

Then in 2005, MOE revised this strategic program and developed “MOE’s Perspectives on Endocrine Disruption-ExTEND 2005-” (ExTEND: Enhanced Tack on Endocrine Disruption). In addition to conducting the necessary survey and research on endocrine disruptors as part of the comprehensive measures to deal with chemicals on the basis of ExTEND 2005, MOE has been making efforts to enhance the information sharing and communication for the better understanding of general public.

In ExTEND 2005, the following comprehensive measures have been promoted to deal with the endocrine disruptor problems:

(1) Observation of wildlife, (2) Evaluation of the environmental concentrations and exposure level, (3) Promotion of basic research, (4) Effects assessment, (5) Risk assessment, (6) Risk management, and (7) Promotion of information sharing and risk communication. Development of strategies for testing and assessment of chemicals is a current important task to be considered.



International Cooperation has also been reinforced through works with OECD under the Endocrine Disruptor Testing and Assessment (EDTA) program and with WHO on “Global Assessment of the State of the Science of Endocrine Disruptors”, bilateral joint research projects with UK and US, and annual international symposia.

It has been five years since the ExtEND 2005 was initiated. MOE plans to review the outcomes under this program and to discuss the issues to be solved regarding the endocrine disrupting effects in the next five years.

This presentation will also outline the chemical management policy in Japan including Chemical Substances Control Law, Pollutant Release and Transfer Register (PRTR), etc. It is a future task for MOE to consider how to include and use the results of testing and assessment of chemicals on endocrine disruptive effects in the risk management system.



Poster Abstracts

- (P1) Comparison of Physicochemical Treatment Options for Water Reuse of Secondary Effluent from Wastewater Treatment Plant
Ilho Kim, Naoyuki Yamashita and Hiroaki Tanaka

- (P2) Occurrence of Pharmaceuticals and Personal Care Products in Yodo River System
Seiya Hanamoto, Hiroki sugishita, Naoyuki Yamashita, Hiroaki Tanaka,
Chie Konishi and Isao Howa

- (P3) Behavior of Pharmaceutical Active Compounds during Sewage Treatment Process: Occurrence, Removal and Distribution in the Dissolved and Suspended Phases
Masanori Narumiya, Takashi Okuda, Norihide Nakada, Naoyuki Yamashita,
Hiroaki Tanaka, Kazushi Sato, Minekazu Sueoka and Toshio Oiwa

- (P4) Developing Methods for Screening Nanoparticle Effects in Fish
Rhys Goodhead, Tessa M. Scown, Okhyun Lee, Blair D. Johnston, Tetsu Kudoh,
Taisen Iguchi, Charles R. Tyler



(P1) Comparison of Physicochemical Treatment Options for Water Reuse of Secondary Effluent from Wastewater Treatment Plant

Ilho Kim, Naoyuki Yamashita and Hiroaki Tanaka

Research Center for Environmental Quality Management, Kyoto University, Japan

In Japan, the amount of reclaimed water in 2005 was about 200 million m³, which corresponds to just 1.4% of total effluent from sewage treatment plants. However, water reuse will be necessary for extending available water resources in the future. It will be very important to consider the health assessment of pathogenic microorganisms, chemical constituents and endocrine disrupters for the reuse of secondary effluent. This study focused on the applicability of O₃, UV/H₂O₂ and O₃/UV processes for water reuse in terms of energy consumption, disinfection effectiveness, the formation potential of DBPs and ecological risk decrease as well as pharmaceuticals removal.

The performance of O₃, UV/H₂O₂ and O₃/UV processes for the pharmaceuticals removal was investigated in this study. Effective pharmaceuticals removal was achieved by the introduction of less electrical energy comparing to other pollutants such as MTBE, BTEX and atrazine, irrespective of the investigated processes. In addition, UV/H₂O₂, O₃ and O₃/UV processes reduced total H/Rs of the 30 pharmaceuticals in tested water by very low level. For O₃ process, O₃ dose of 6 mg/L (O₃ consumption : 4.4 mg/L) was necessary for the effective removal of the 37 pharmaceuticals in secondary effluent. About 4 log inactivation of total coliform can be expected under this operational condition. Consequently, it is expected that O₃ dose of 6 mg/L can meet the guideline for water reuse of Japan and the water quality required for restricted urban and agricultural (non-food crops) reuses in the California Title 22 criteria. The formation potential of bromate is likely to be high under O₃ dose of 6 mg/L. Therefore, the combination of UV or H₂O₂ with O₃ process is recommended to suppress bromate formation, for direct and indirect potable reuses. In addition, UV/H₂O₂ process can be a treatment option in terms of bromate suppression.

UV/H₂O₂ (UV dose : 923 mJ/cm², H₂O₂ : 6.2 mg/L) and O₃/UV (O₃ consumption : 3.4 mg/L (O₃ dose : 4 mg/L), UV : 1,846 mJ/cm²) processes can be expected to achieve more than 5log inactivation of total coliform. Moreover, no bromate will be formed during UV/H₂O₂ process, and the combination of UV with O₃ process also can suppress the formation of bromate by blocking the reaction of residual O₃ with Br through the photodegradation of residual O₃. Therefore, the processes can be applied for unrestricted urban reuse, agricultural reuse (food crops) and recreational reuse as well as restricted urban and agricultural (non-food crops) reuses of sewage water although much more energy consumption will be necessary comparing to for O₃ process. On the other hand, decrease effect of ecological risk before and after treatment was evaluated for the parent pharmaceuticals in this study. As a result, it was observed that ecological risk caused by the parent pharmaceuticals could be reduced considerably after treatments, irrespective of the applied processes. Therefore, the investigated processes can be used as treatment options for indirect potable reuse, recreational reuse and groundwater reuse etc of sewage water in terms of ecological risk decrease for the parent pharmaceuticals, although the adverse effect by by-products are still questioned.



(P2) Occurrence of Pharmaceuticals and Personal Care Products in Yodo River System

Seiya Hanamoto¹, Hiroki sugishita¹, Naoyuki Yamashita¹, Hiroaki Tanaka¹, Chie Konishi² and Isao Howa²

¹Research Center for Environmental Quality Management, Kyoto University, Japan
and ²Murata Keisokuki Service Co., Ltd., Japan

In recent years, pharmaceuticals and personal products (PPCPs) have been increasingly detected in wastewater and surface water. Due to their bioactive nature and potential environmental risks, PPCPs are recognized as a group of emerging contaminants. Field surveys in the Yodo River System that supplies drinking water for more than 16 million and receives wastewater from more than 4 million in Japan were conducted four times in spring, summer and fall. Grab water samples were collected in glass bottles from 36 points including the outlets of 7 wastewater treatment plants (WWTPs). The samples were preserved in the ice immediately after adding 1 g/L of each EDTA-Na₂ and ascorbic acid. Each sample was filtrated through a glass fiber filter (Whatman, GF/F) and extracted through an Oasis HLB plus cartridge (225mg, Waters). For the analysis by LC-MS/MS, we employed the simultaneous quantification method for PPCPs developed by Konishi et al. (2006). The analytes were eluted by methanol then the eluents were dried under stream of nitrogen gas and redissolved with acetonitrile and 0.1 % formic acid as 7:93 before the injection into LC-MS/MS. For the analysis by GC-MS, the analytes were pretreated in the same manner except that the solvent used in elution and redissolution was dichloromethane and that the analytes were derivatized prior to the injection.

In the surveys(2007,November) 107 PPCP were monitored, 70 were found from discharge from municipal wastewater treatment plants(WWTPs) and/or river waters at the maximum concentration of 1829 ng/L. Galaxolide, Sulpiride, Crotamiton were detected in more than 1000ng/L in more than three WWTPs effluent (out of seven). Clarithromycin, tonalide, disopyramide, ketoprofen were detected in more than 400ng/L in more than three WWTPs effluent (out of seven). Caffeine was detected in more than 1000ng/L in some tributaries. The results suggested that the WWTPs were a major source of many PPCPs to the rivers. However, some were detected at upstream of the rivers or in tributaries where no WWTP discharge were received, indicating the existence of other sources than the WWTPs.



(P3) Behavior of Pharmaceutical Active Compounds during Sewage Treatment Process: Occurrence, Removal and Distribution in the Dissolved and Suspended Phases

Masanori Narumiya¹, Takashi Okuda¹, Norihide Nakada¹, Naoyuki Yamashita¹, Hiroaki Tanaka¹, Kazushi Sato², Minekazu Sueoka² and Toshio Oiwa²

¹Research Center for Environmental Quality Management, Kyoto University, Japan, and ²Teijin Eco-Science Co., Ltd., JAPAN

With the development of analytical equipments, trace contaminants such as endocrine disrupting chemical has been detected in the aquatic environment. In recent years, pharmaceuticals and personal care products (PPCPs) have attracted increasing attention as emerging contaminants. The presence of PPCPs in the aquatic environment could lead to serious problems such as adverse effects on aquatic ecosystem because many of them are highly bioactive. To reveal the current status of contamination by PPCPs, field surveys have been conducted in the aquatic environment by now. As a result of the surveys focused on sewage treatment plants (STPs), effluents from STPs were found to be the main sources of PPCPs in rivers. Therefore, in order to improve the removal efficiency of PPCPs in STPs, it is necessary to make clear the removal mechanism during sewage treatment process. However, there is still a lack of data about the occurrence and fate of PPCPs during the sewage treatment process especially in consideration of both dissolved and suspended phases. The objective of this study is to understand the behavior of PPCPs in consideration of suspended phase during the sewage treatment process. To achieve this objective, we developed the analytical method of PPCPs in sewage sludge samples and then conducted surveys in 5 STPs in Japan. Sixty-eight target compounds were simultaneously analyzed by liquid chromatography-tandem mass spectrometry. As a result of the survey, 66 compounds out of 68 were detected in sewage samples. In suspended phase of sludge samples, 60 compounds were detected between $\mu\text{g}/\text{kg-dry}$ to $10 \text{ mg}/\text{kg-dry}$ levels and this level was nearly close to or less than reported data from foreign countries. Removal efficiency of target compounds ranged between under 0% to around 100% and some target compounds were difficult to be removed at activated sludge process. Based on our calculation, biodegradation was the main removal mechanisms for target compounds expect for triclosan and levofloxacin, which were removed by withdrawal of sludge.



(P4) Developing Methods for Screening Nanoparticle Effects in Fish

Rhys Goodhead¹, Tessa M. Scown¹, Okhyun Lee¹, Blair D. Johnston¹, Tetsu Kudoh¹, Taisen Iguchi², Charles R. Tyler¹

¹Environmental and Molecular Fish Biology, Hatherly Laboratories, University of Exeter, Prince of Wales Road, Exeter. EX4 4PS, United Kingdom and ²National Institute for Basic Biology, Nishigonaka 38, Myodaiji, Okazaki 444-8585 Aichi

Many studies have been conducted to assess the biological effects of engineered nanoparticles (ENPs) via airborne and dermal exposure on human health. Despite this, there are still many uncertainties regarding their potential toxicity. Even less is known on the potential hazards posed by ENPs to aquatic organisms. The aquatic environment is likely to receive an ever increasing burden of ENPs as the range of materials and the variety of their applications increase. Reliable and costs effective methods of screening for toxicity and health effects on aquatic ecosystems have yet to be devised. Our investigations into the use of a fish hepatocyte cultures as a screen for assessing cellular uptake and toxicity of ENPs, found that this model lacked the required sensitivity for biomarkers most likely associated with ENP effects, such as those for oxidative stress (Scown, Goodhead et al., 2009, submitted). We have therefore now progressed to investigating the potential use of zebrafish embryos, an easily manipulated model developmental organism, to screen for health effects of ENP exposure. We (and others) have under taken exposures of zebrafish embryos and later (early) life stages via the water but effects are rarely seen below concentrations in the mg/l range. These studies, however, are complicated by the fact that many of the ENPs studied aggregate in the water/exposure medium and sediment out, with few studies quantifying what material (nano, or otherwise) is taken through the chorion into the embryo. This makes it extremely difficult to assess whether materials in their nano form have more harmful effects *per se* compared with their bulk counterparts. To avoid issues related to quantifying ENP uptake we are now conducting a series of direct micro-injection studies into zebrafish embryos with metal and metal oxide based ENPs and searching for their potential to induce effects on a range of developmental endpoints. Using standard developmental assays, embryos are being assessed 1, 2, 4, 6, 8, 12, 48 and 96 hours post injection, for effects on survival, abnormalities in growth, time to hatching and heart rate. Subsequent analysis will include targeted in situ hybridization and rtPCR for expression of genetic markers associated with metal toxicity (metallothionein, MT2) and xenobiotic breakdown. These investigations have recently been started using well characterised ZnO and Ag nanoparticles. Adopting a microinjection approach we hope to be able to develop a high throughput system that will allow rapid assessment of novel ENPs likely to be put into high production and potentially have a significant presence in the aquatic environment.

Scown, T.M., Goodhead, R., Johnston, B.D., Moger, J., J.R. Lead, vanAerle, R., Iguchi, T., Tyler, C.R. (2009). An assessment of cultured fish hepatocytes for screening cellular uptake and (eco)toxicity of nanoparticles. **Environmental Chemistry** *submitted*



Talk Slides

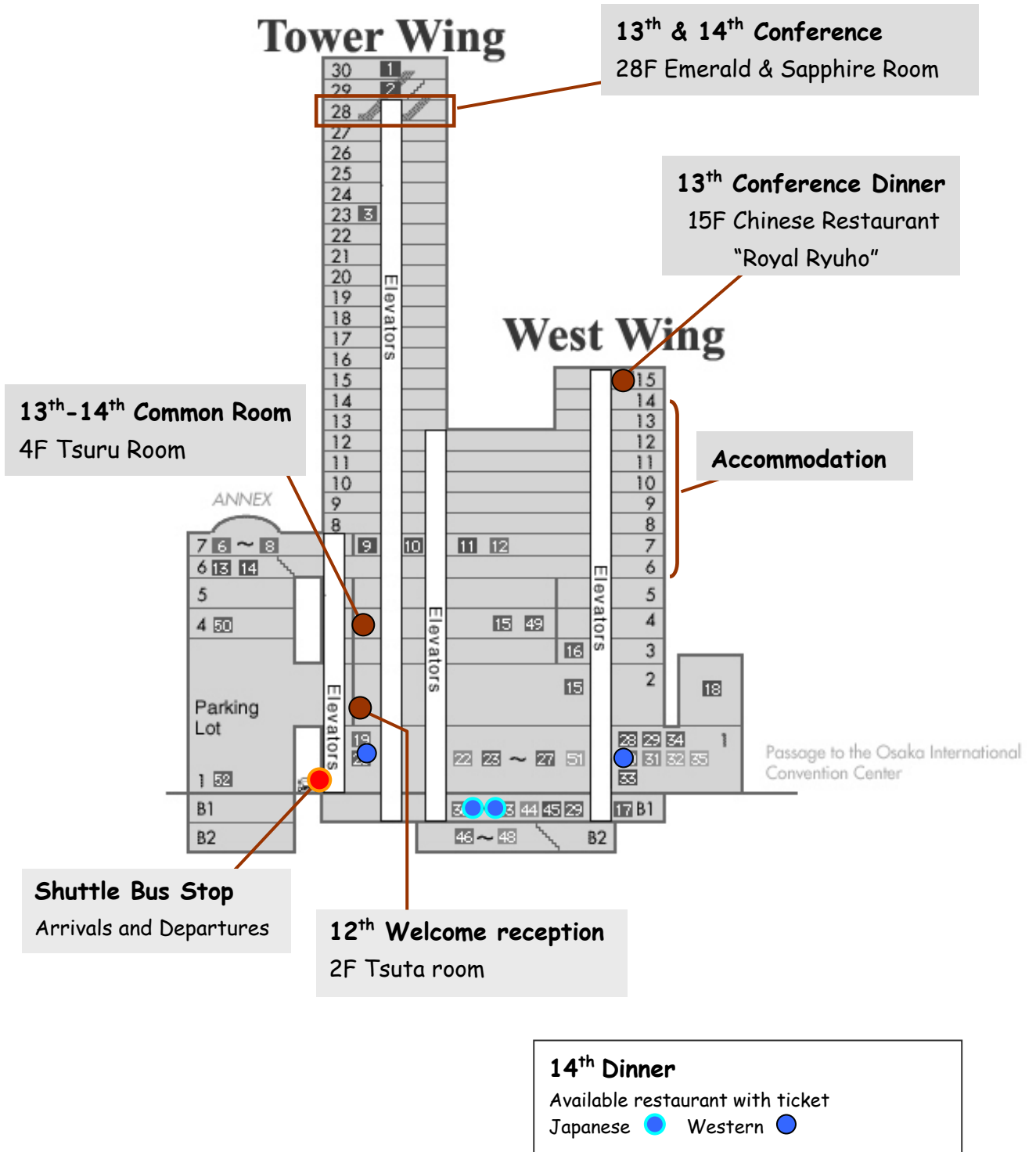


Publication Lists

September 2008 – October 2009

MAPs

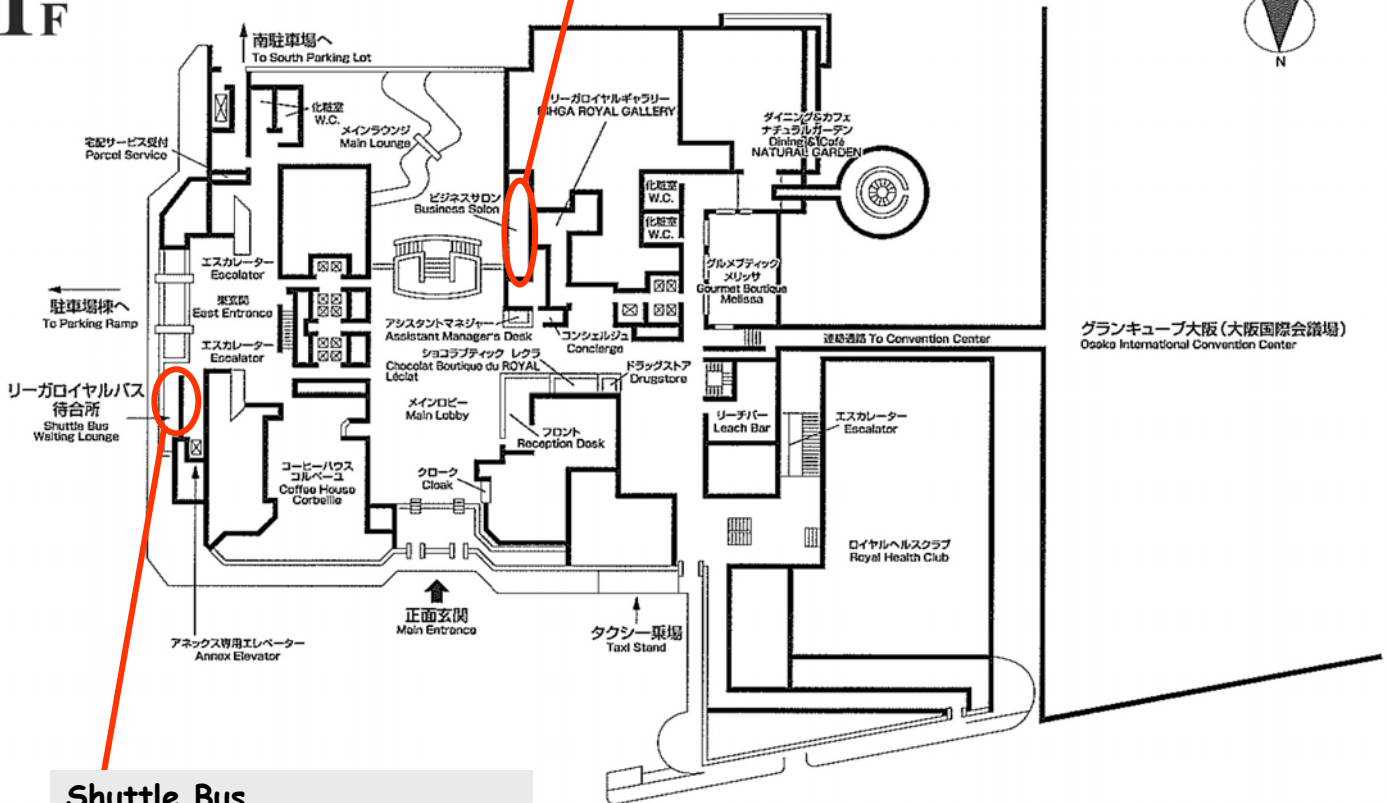
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Business salon 8:00 to 21:00,
 Internet: free for the first hour
 500 yen for each additional hour

1F



Shuttle Bus
 Arrivals and Departures
 To JR Osaka station
 To Yodoyabashi station
 (First bus at 7:30, last bus at 22:00)

Conference Room

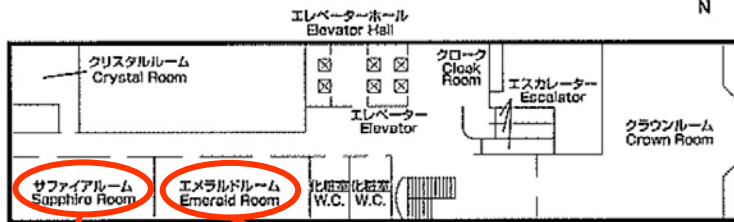
13th Conference : Tower Wing 28F "Emerald Room"
9:00- Opening

14th Conference : Tower Wing 28F "Sapphire Room"
8:40- Opening
12:15- Signing ceremony

Common Room : Tower Wing 4F "Tsuru Room"
13th 8:00 - 24:00
14th 8:00 - 14:00
Printer, copy machine are available, but NOT internet.
Contact us when you need to use this room.

4F Tsuru Room
(13th-14th Common Room)

28F(Tower Wing)



28F Sapphire Room (14 th Conference Room)	28F Emerald Room (13 th Conference Room)
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4F(Tower Wing)



Conference Dinner

13th Conference dinner
19:00- 21:00 West Wing 15F Chinese Restaurant "ROYAL RYUHO"