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Workshop 3.2

Search for the evidence of endocrine disruption in the aquatic environment: Lessons to be learned from joint biological and chemical monitoring in the European Project COMPREHEND*

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Abstract: Between January 1999 and December 2001, the European Community project COMPREHEND was performed. The overall aim of COMPREHEND was to assess endocrine disruption in the aquatic environment in Europe, consequent to effluent discharge, with emphasis on estrogenic activity. COMPREHEND demonstrated the widespread occurrence of estrogenic effluents across Europe and presented evidence of impacts on a range of wild fish species. Using a variety of bioassays in combination with chemical analytical methods, estrogenic steroids of human origin from domestic wastewater effluents were identified as the most pervasive problem, although alkylphenols may be important estrogenic components of some industrial effluents. New tools have been developed for the identification of estrogenic effluents, and recommendations are made for the improvement of existing techniques. We have shown that individual fish within natural populations may be feminized to varying degrees, but it has not been possible to show, using traditional fish population parameters, that the survival of fish populations is threatened. However, laboratory-based fish life-cycle studies demonstrate the sensitivity of fish to estrogen (and androgen) exposure and

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how this might lead to complex (and potentially damaging) genetic changes at the population level. New approaches to this problem, utilizing recent advances made in the field of molecular and population genetics, are recommended. Finally, a study of estrogenic and androgenic activity of waste waters during the treatment process has shown that some of the existing wastewater treatment technologies have the potential to eliminate or minimize the hormonal activity of the final effluent.

In the period between January 1999 and December 2001, the community program of research on endocrine disruptors and environmental hormones (COMPREHEND), a European Community-financed project (ENV4-CT98-0798), was performed. In COMPREHEND, coordinated by Prof. Alan D. Pickering, 12 partners from 7 European countries were involved. COMPREHEND was designed with the overall aim of assessing the evidence for endocrine disruption in the aquatic environment in Europe, consequent to effluent discharge. Particular emphasis was given to the estrogenic activity of both domestic and industrial effluents and their impacts on fish (both freshwater and marine). The overall objectives of the COMPREHEND program were to: (1) examine the occurrence and distribution of endocrine-disrupting effluents across a range of European countries using existing fish-exposure techniques for the detection of reproductive interference, (2) analyze those effluents which are shown to be capable of interfering with the fish endocrine system for the principal causative agents, (3) investigate the influence of partitioning within the water column of known endocrine disrupters in relation to their impact on fish, (4) assess available evidence and collect new information on the impacts of endocrine disruptors on aquatic wildlife, (5) develop and improve tools (both in vivo and in vitro) for the rapid detection of endocrine disruptors and investigate the application of existing in vitro techniques for the direct screening of complex effluents. In this manuscript, the lessons learned and suggestions for future activities in this field are described. Many more details can be found in published data or upcoming publications from the laboratories involved.

COMPREHEND has shown that estrogenic effluents are widespread across Europe. Approximately one-third of the municipal sewage treatment works (STW) effluents examined during the 3-year program were found to be strongly estrogenic and capable of stimulating vitellogenesis in juvenile or male fish (from a range of species) after a 2–3 week exposure period. Several industrial wastewater effluents (principally those involved with chemical/pharmaceutical manufacture) were also found to be significantly estrogenic to exposed fish. However, other municipal STW effluents and industrial waste waters were apparently nonestrogenic or only weakly estrogenic. This variability in estrogenic activity was detected by both in vivo and in vitro techniques and was characteristic of effluents from most countries examined during the survey. We conclude that strongly estrogenic municipal effluents will be reasonably commonplace across mainland Europe (as they are in the United Kingdom) and, therefore, there is the potential for estrogenic endocrine disruption of aquatic wildlife in all countries (the situation with industrial effluents will depend very much upon the nature of the industrial processes). National sewage effluent surveys have already been undertaken in the United Kingdom, the Netherlands (as part of the LOES/COMPREHEND program), and in Sweden. The Swedish survey was initiated as a direct result of the Swedish involvement (three laboratories) in the COMPREHEND program and demonstrates how EU funding may act as a lever to secure additional national funding for environmental research and monitoring programs. We are also aware of additional effluent survey work in France (indeed, we invited environmental scientists from Cemagref to all the COMPREHEND meetings and they were able to participate in all but one of the six planning/reporting meetings), in Austria and, most recently, in Switzerland. Several of the COMPREHEND senior scientists have been involved in the peer-review process of these new initiatives and one of the COMPREHEND partners (EAWAG) is heavily involved in the Swiss national program. Thus, an increasing number of European countries are now investing significantly in studies on aquatic endocrine disruption.

The in vivo approach to measuring effluent estrogenicity, by measuring induced vitellogenin (VTG) levels in the blood of immature fish (rainbow trout, carp, and cod) exposed in situ to the effluent was difficult to control and standardize. This approach, whilst being a useful indicator of estrogenicity, is not suitable for the routine assay of effluents within any statutory framework. Inconsistencies were found in the current methods for blood plasma vitellogenin analyses. Enzyme-linked immunoabsorbent assay (ELISA) techniques are preferable to radioimmunoassay, in terms of the facilities required and their ease of operation, but most existing ELISA techniques lack the sensitivity of radioimmunoassay (RIA). However, the only homologous rainbow trout ELISA tested during COMPREHEND did have the required sensitivity, and we recommend that future assays use homologous systems wherever possible. A further problem was the lack of an agreed VTG standard for the rainbow trout (or for any other species), and an interlaboratory comparison of existing "purified" VTG preparations is urgently required.

Several in vitro assays (human, yeast, and fish cell-based) for the direct measurement of estrogenic activity were tested for use on complex effluents. Sample preparation, in most systems, consisted of solid-phase extraction and elution, but did not include further fractionation. There were marked differences in sensitivity of the in vitro assays, but specificity to known estrogens was reasonably consistent, irrespective of the nature of the assay. Thus, in vitro systems based upon fish estrogen receptors produced similar estimates of estrogenic activity in effluents to those based on human estrogen receptors. Assays based on genetically modified yeast cells (the YES and YAS assays) were sufficiently robust and reliable for most purposes and were considerably simpler to perform than those assays requiring sterilized cell-culture techniques. If insensitivity is a problem, we recommend that effort is spent increasing the sensitivity of the yeast cell systems. The YES assay does have the potential for standardization in routine effluent testing, but COMPREHEND identified two sources of possible interference. Toxicity of the effluent to the yeast cells can, in some cases, be overcome by dilution but suppression of the estrogenic response at the level of the receptor requires that the assay protocol is modified, in order to detect such effects. It was demonstrated that alkylphenol acetates suppress the activity of estrogenic steroids and of alkylphenols. The point must be made, however, that in vitro studies are not a substitute for in vivo studies. We have shown, for example, that EE2 is approximately ten times more estrogenic to zebrafish than is E2, whereas most in vitro assays failed to separate their relative potencies.

The techniques for chemical analysis of alkylphenols and related compounds and of bisphenol A were reliable and repeatable (at the $\mu g\ l^{-1}$ concentration), but problems were experienced with the measurement of estrogenic steroids (at the $ng\ l^{-1}$ concentration) in such complex matrices. Bisphenol A was detectable in municipal effluents, but at concentrations less than 5 $\mu g\ l^{-1}$ and nonylphenol, the most abundant of the alkyl phenols, was generally below 2 $\mu g\ l^{-1}$. Estrone (E1) measurements were the most consistent in terms of recovery, and a good correlation was obtained in a comparison of the techniques in two laboratories for measurements of the same set of wastewater samples. There was poor agreement with estradiol (E2) measurement, and both laboratories experienced very poor recoveries with estriol (E3) and low sensitivity with ethinylestradiol (EE2). Estrone measurements in STW effluents showed a good degree of correlation with estrogenic activity (as measured with in vitro assays) and estrogenic steroids E1 and E2 were generally in the 0 to 10 $ng\ l^{-1}$ range. EE2, however, was often at or below the limit of detection (approximately 1 $ng\ l^{-1}$). We recognize the need for an interlaboratory comparison of analytical techniques for steroids in complex effluents. It may be necessary to develop new analytical approaches, and the role(s) of RIA needs to be considered alongside more traditional analytical chemistry. Estrogenic steroids were generally below the limits of detection for most industrial waste waters (unless there was a significant component of the effluent originating from domestic/human sources within the industrial plant). The strong estrogenicity of two industrial effluents (specialty chemicals manufacture and textile) correlated with relatively high levels (up to 5 $\mu g\ l^{-1}$) of nonyphenol (NP) and nonylphenol ethoxylates (NPEs) and, in the effluent from another chemical manufacturing plant, the toxicity identification and evaluation (TIE) approach identified a hydroxyphenyl hexanoic acid as the principal estrogenic contaminant. The highest level of bisphenol A (BPA) (1.14 μg l^{-1}) was found in the effluent from a pharmaceutical plant.

TIE identified E2, E1, and EE2 as the principal estrogenic components of domestic raw sewage, with EE2 and E1 dominating the estrogenic activity of the final effluent. Taking into consideration the potencies of the various estrogenic compounds measured in municipal STW effluents, we conclude that natural and synthetic steroids, of human origin, are by far the most important estrogenic components and are responsible for most of the estrogenic effects seen in vivo and in vitro. EE2 may be particularly important in this respect, but the limitations of our current analytical techniques are a major constraint to confirming the importance of this component of the contraceptive pill. TIE also provided evidence of "cooperative" effects between the different steroids, making the measured activity (YES assay) approximately three times greater than the sum of the activity of the individual components. Currently, there is no consensus on the best approach for experimental design and data analysis to measure potential interaction between estrogenic compounds in complex mixtures. This is clearly an important area and one which requires more research. In this context, we are pleased that two new, EU-funded research programs (ACE and EDEN) will focus primarily on the effects of mixtures of endocrine disruptors. Several of the original COMPREHEND consortium are participants in the EDEN program (under Framework V) and will address the issue of mixtures in the natural environment.

Laboratory studies demonstrated that natural suspended sediments in the water do not modulate, to any significant extent, the estrogenicity of octylphenol to fish and it is concluded that the most important route of exposure for both alkylphenols and steroids is directly from the water, rather than indirectly from contaminated suspended sediments or via the food chain. However, a Dutch survey of the distribution of potential xenoestrogens in the aquatic environment found alkylphenols and their ethoxylates at particularly high concentrations in suspended, particulate matter in fresh water. Whether such material is biologically available to fish requires further study, as does the role of bed sediments as a potential route of exposure for truly benthic/burrowing animals.

Strong androgenic activity (YAS assay) was found in the influent to domestic STWs and was presumed to be of human origin, but most of the activity disappeared during the wastewater treatment process. Androgenicity (and some estrogenicity) was also detected in some pulp-mill effluents when zebrafish were exposed to effluents dilutions or to wood sterols in the laboratory, but the environmental consequences of this are unknown.

Thus, COMPREHEND has shown that hormonally active substances (primarily steroids) are being discharged into the aquatic environment at concentrations initially high enough to induce estrogenic responses in experimentally exposed fish. However, in most circumstances the effluents are rapidly diluted following discharge and, therefore, the question arise as to whether native fish in the receiving waters are adversely impacted. Samples of wild fish, taken in the vicinity of some of the known estrogenic municipal effluents were found to have abnormally high levels of VTG (a female egg protein) in the blood of juvenile or male fish. Moreover, increased levels of intersexuality (presumed males with oocytes present in the testis) were found in the same vicinity. The species of wild fish showing such evidence of estrogen exposure were the common bream, the common carp, the roach, and the gudgeon, all belonging to the carp family (the Cyprinidae). There was no evidence that the gudgeon (a benthic species) was significantly more affected than the roach (a more pelagic species). Exposure of fertilized brown trout eggs to rivers impacted by sewage effluents resulted in significant impacts on embryonic development, but it is too early to say whether these were mediated in any way by endocrine disruption. Nevertheless, it is interesting that some of the novel, estrogen-sensitive gene products identified as part of COMPREHEND had strong homologies with growth and transcription factors. The role of such proteins in (and possible estrogenic influences on) normal cellular development is an area that merits further research.

All the evidence obtained thus far is indicative of localized areas of estrogenic impact on individual fish, but it tells us little about the longer-term consequences at the population level. An analysis of one of the largest freshwater fish population data sets (for bream in the Netherlands) available to science revealed only limited circumstantial evidence of possible impacts of endocrine disruption at the population level. Thus, any significant deviation of sex ratios in bream populations away from a presumed normality of 50/50 was always in favor of females. In addition, evidence was found in one population of a significant decline over 30 years in the testis size of sexually mature male fish, whereas the female fish in the same population showed no change in gonad weight. However, we recognize that many factors might influence sex ratios in fish populations (and, indeed, relative gonad size) and accept that the analytical approach to fish population parameters adopted during COMPREHEND can never show cause and effect. Moreover, the size of cyprinid fish populations in particular are often determined by stochastic factors, such as food availability during the early life stages, and these may mask any effects of endocrine disruption on population structure. We are of the opinion that a new approach to this problem is required and recommend the development of reliable tools for the genetic sex determination of fish and the use of population genetics to investigate the potential impacts of endocrine disruption. If, for example, a population is being maintained by just a small percentage of the sexually mature adults, it might be possible to recognize this in terms of a reduction in genetic diversity within the population. Such a situation might not have an immediate impact on fish numbers, but it might make the population more susceptible to other environmental stresses, by limiting the genetic diversity available for future natural selection.

In view of the difficulties inherent in any study of natural fish populations exposed to endocrine disrupting influences, we also examined impacts of known estrogens and of wastewater effluents on fish (primarily zebrafish, with some work on the stickleback) held under laboratory conditions, including studies of chronic exposure over two complete generations. Chronic exposure to as little as 0.6 ng l^{-1} EE2 (below the limits of chemical detection for most effluents) was sufficient to sex-reverse male fish, and 1.5 ng l^{-1} stimulated vitellogenesis in juvenile fish. The stickleback studies indicated a particularly sensitive window of exposure to estrogens during the first two weeks post-hatch. Sex reversal in the opposite direction could be induced by low-level androgen (methyltestosterone, MT) exposure but higher concentrations of MT caused feminization—a clear case of a U-shaped dose–response curve. The feminizing effects of MT were also observed in a study of natural sex reversal in the cuckoo wrasse. One of the more unusual results from chronic exposure studies was the effect of wood sterols (and pulp-mill effluent) on the sex ratios of successive populations of zebrafish. The first progeny (F1) of the chronically exposed fish were predominantly male but then reversed in the F2 generation to predominantly female. It is possible that this effect may be caused by the successful spawning of sex-reversed, F1 genetic females. Whatever the explanation, more work is now required into determination of the genetic sex of individual fish and the reproductive competence of sex-reversed fish.

COMPREHEND was unable to find any evidence of endocrine disruption in a brackish-water crustacean *Nitocra spinipes* exposed to estrogenic substances, and we question whether this species has a functional estrogen receptor. Some evidence of ecdysteroid receptor antagonism was found for a waste water effluent from a chemical manufacturing plant but we are not aware of any evidence of problems in aquatic invertebrates consequent to such endocrine disruption.

Whenever the estrogenic activity of the raw sewage (influent) was measured (using in vitro techniques) it was found to be strongly estrogenic. The fact that some of the final effluents were nonestrogenic implies that some existing STWs have the capacity to remove most, if not all, of the estrogenic activity. Similar observations also apply to androgens in municipal STWs. Chemical analyses and TIE indicated that the estrogenic activity in the raw sewage was caused by E2, E1, and EE2. E1 to E2 ratios were much higher in the final effluent, indicating conversion of E2 to E1 during the treatment process, but EE2 may be the dominant estrogenic component of the effluent (difficult to confirm in the unfractionated effluent because of insufficient sensitivity in the EE2 analysis). A comparison of different types of STWs and a study of estrogenicity during the various stages of treatment confirmed that the majority of the activity was lost during secondary biological treatment. Indeed, in systems with primary treatment only, androgenic and estrogenic activity could actually increase, presumably because of deconjugation. A major factor determining the estrogenicity of the final effluent was the residence time during the treatment process, with effluents from treatment plants with residence times over 15 h having minimal activity. Thus, the solution to this potential environmental problem may already exist within the existing technology. However, alternative technologies, aimed to avoid the release of estrogenic compounds in the waste streams already at the source, must be seriously analyzed as well.

In summary, the following conclusions may be drawn from the COMPREHEND program.

- Estrogenic wastewater effluents (both domestic and industrial) are widespread across Europe.
- Steroids are the principal estrogenic compounds in municipal STW effluents.
- Improved analytical techniques for the measurement of estrogenic steroids in complex matrices are urgently needed.
- Nonylphenol may play a significant estrogenic role in some industrial effluents.
- For in vivo screening, fish vitellogenin assays need to be improved and standardized.
- Current in vitro screening techniques can be used to assess the estrogen receptor-mediated estrogenicity and the androgen receptor-mediated androgenicity of unfractionated effluents.
- We need increased molecular mechanistic understanding of endocrine disruption. This as a basis for proper risk assessment and the development of novel bioanalytical tools.
- The principal xenoestrogen exposure route for freshwater fish is directly from the water.
- There is evidence of endocrine disruption in individual fish from wild stocks in the vicinity of estrogenic effluents.
- We need a new approach (using molecular genetic techniques) for the study of impacts at the population level.
- Sex reversal in either direction is a possible consequence of exposure to hormonally active compounds.
- Chronic exposure to some effluents can cause masculinization in the F1 generation, followed by feminization in the F2 generation.
- We could not find evidence of crustacean sensitivity to xenoestrogens.
- Existing wastewater treatment technologies have the *potential* to eliminate most of the estrogenic and androgenic activity before discharge to the environment.
- Technologies aimed at avoiding the release of endocrine active compounds in the waste stream might be an alternative.

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