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Summary and conclusions

Supplementary investigation of estrogens in the aquatic environment

To supplement the 2004 national survey of estrogens in the aquatic environment in Denmark some targeted additional investigations were carried out during 2005. The investigations included small streams receiving discharges of sewage treatment plant effluents, effluents from septic tanks at farms or other isolated dwellings, and drainage water from fields amended with either liquid manure or sewage sludge. There was none or very little estrogenic activity in the stream water samples whereas the sediments had levels of activity that clearly showed that they were affected by the discharges. The estrogenic activity in septic tank effluents was, on the other hand, high, and a significant dilution is required to reduce the activity to below known biological effect levels. Only very low or even no estrogenic activity was found in the investigated field drains from manure or sludge amended fields.

Background and objectives

A comprehensive survey of the estrogenic activity in freshwater environments in Denmark was carried out in 2004 and published by the DEPA in 2005 as Environmental Project No. 977. The results of the survey showed that the estrogenic activity in streams and lakes unaffected by sewage effluent discharges was generally low while the activity in other streams and lakes was somewhat higher but still in most cases below the biological effect threshold. Sewage treatment plants that comply with the requirements of the Danish Water Action Plan regarding removal of organic matter and nutrients generally also performed well with regard to removal of estrogenic activity and so did biological sand filters. In contrast to this, the removal of estrogens in small traditional sewage treatment plants was often poor.

Though the survey did not indicate estrogenic activity to be a significant and widespread problem in Danish freshwater environments there were a number of issues that were not elucidated sufficiently to enable clear conclusions to be drawn. Examples are: discharges of drainage water from fields amended with either liquid manure or with sewage sludge, septic tank effluents from farms and other dwellings outside urban areas, and the effect of certain sewage effluent discharges on the water and sediment quality in small streams where the effluent volume makes up a significant fraction of the total flow in the stream. It was therefore decided to carry out some supplementary investigations of these issues.

The investigation

The supplementary investigation of estrogenic activity in the aquatic environment comprised the following main elements:

- Quality of water and sediment in small streams upstream and downstream of discharges from small, traditional sewage treatment plants as well as of the effluents themselves. Six sites were selected for the investigation and each was sampled only once (spot sampling).

- Levels of estrogens in the effluents from septic tanks at farms and other isolated dwellings in the open land, which are often discharged into field drains through which they end up in streams and rivers. 3-4 24 hours samples were taken at each of the 5 selected sites.

- Estrogenic activity in drainage water from fields onto which either liquid manure or sewage sludge had been amended in the spring of 2005. The sampling was carried out at four locations (three with manure and one with sludge) over a period of 3 weeks starting shortly after the amendment had taken place.

All the samples were tested biologically with the YES assay to determine the total activity of free estrogens while selected samples were additionally analysed chemically for their content of the most important steroid estrogens.

The main conclusions

The supplementary investigations of small streams did not reveal any increase of estrogenic activity in the water phase downstream the points of discharge from traditional sewage treatment plants even though the discharges constituted some 10-20% of the total flow in the streams. It should be noted, though, that in the effluent samples taken concurrent with the stream samples the estrogenic activity was only significant in two out of six samples while very low activity was detected in another two. However, the chemical analyses of downstream sediment samples did show that emission of estrogens apparently occur regularly as there was a significant content of steroid estrogens in these samples.

In the 2004 survey of estrogenic activity in the aquatic environment the variation in estrogenic activity in effluents from traditional sewage treatment plants was also significant and, thus, it is assumed that generally the variation in the performance of the plants within this category is quite significant.

The effluents from septic tanks at farms/houses in the open land did also exhibit considerable variation in the levels of estrogenic activity though in the vast majority of samples the activity was at least 10-100 times higher that the threshold levels for biological effect. This implies that a significant dilution is required prior to or immediately after discharge into streams and rivers to reduce the activity to below the level of concern. It is assessed that this in general is possible to achieve.

The estrogenic activity in drainage water from manure or sludge amended fields determined by the bioassay was found to be below the limit of detection in all samples but one while by supplementary chemical analysis of a few samples low levels of steroid estrogens were detected in 3 out of 5 samples. In the 2004 survey slightly higher, but still rather low levels of estrogens were found in this type of samples. It is assessed that an investigation with a considerably longer duration is required if a more firm conclusion regarding the extent of leaching of estrogens from manure/sludge amended fields shall be drawn.

The project results

The results of the biological tests and chemical analyses of water and sediment from small streams receiving treated sewage from small traditional sewage treatment plants, and of the effluents from these, are shown in Table 1. The effluent and stream water samples were taken by qualified spot sampling (i.e. each sample being composed of five sub-samples), and the downstream samples of both stream water and sediment were taken at a distance from the discharge point corresponding to approximately 10 times the width of the stream to ensure a proper mixing of the stream water with the effluent.

Table 1
Estrogenic activity in WWTP effluents, receiving streams and sediments. Results in E2 equivalents (ng/L for water and ng/kg dw for sediment))

WWTP	Test type	Effluent	Receiving stream, water		Receiving stream, sediment	
			up	down 1	up	down 1
W1	biological	neg	neg	neg	-	neg
	chemical	-	-	<0.1	-	280
W2	biological	19.8	neg	neg	neg	neg
	chemical	-	-	0.19	200	30
W3	biological	neg	neg	neg	neg	510
	chemical	0.14	-	0.21	100	910
W4	biological	neg	0.37	neg	-	110
	chemical	0.38	-	0.22	-	420
W5	biological	neg	neg	neg	-	neg
	chemical	-	-	<0.1	-	190
W6	biological	3.94	0.36	neg	-	1160
	chemical	-	-	0.10	-	810

- = not analysed

The estrogenic activity in septic tank effluents from farms/houses in the open land was studied at five locations, and at each of them 24 hour samples were taken 3-4 times during a week. The main results are presented in Table 2. Only in one case did some of the activity originate from the synthetic estrogen used in contraceptive pills. The level corresponded to about 10 ng E2/liter.

Table 2
Estrogenic activity in septic tank effluents. Results in E2 equivalents (ng/L).

Septic tank	Biological testing		Chemical analysis*
	Average	Range	
S1	88.2	55.1 - 125	84.1
S2	425	352 - 425	417
S3	28.5	24.5 - 31.5	12.2
S4	44.2	29.5 - 68.9	17.4
S5	4.93	nd - 14.8	4.16

* only the sample with the highest biological activity was analysed chemically
nd = not detected

The samples of drainage water from manure and sludge amended fields (three manure and one sludge field) were taken in May 2005 by continuous sampling during 2-3 or 3-4 days per week over a total period of 3 weeks (one site was sampled for only one week).

Only in one sample out of a total of 20 a weak estrogenic activity (0.1 ng E2/L) was detected by the bioassay while in 3 out of 5 samples that were analysed chemically a content of steroid estrogens was detected. The levels were, however, quite low - from 0.1 to 0.32 ng E2/liter.

Sammenfatning og konklusioner

Supplerende undersøgelser af østrogener i vandmiljøet

Der er foretaget en række undersøgelser som supplement til kortlægningen fra 2004 af østrogener i vandmiljøet. Undersøgelserne har omfattet små vandløb med udledning af spildevand fra små, traditionelle renseanlæg, afløbsvand fra septiktanke samt drænvand fra marker, hvor der er udbragt gylle eller spildevandsslam. Der kunne ikke påvises nævneværdig aktivitet i vandløbsprøverne, hvorimod sedimentet havde et ikke ubetydeligt indhold af østrogener. Den østrogene aktivitet i afløbsvand fra septiktanke var derimod gennemgående høj og vil kræve betydeligt fortynding for at nå ned under et niveau, hvor der er påvist biologiske effekter. Der blev kun fundet meget lav eller ingen østrogen aktivitet i de undersøgte drænvandsprøver.

Baggrund og formål

I 2004 blev der gennemført en omfattende kortlægning af østrogenaktiviteten i danske ferskvandsmiljøer samt i mulige kilder til belastning af vandmiljøet med østrogent virkende stoffer. Kortlægningen, der blev publiceret i starten af 2005 som Miljøprojekt nr. 977, viste, at den østrogene aktivitet i vandløb og søer, der ikke modtager spildevand, generelt var lav, mens aktiviteten i øvrige vandløb som gennemsnit var en smule højere, men dog stadig kun i mindre omfang over den p.t. skønnede nedre grænse for biologiske effekter. Renseanlæg, der er udbygget i overensstemmelse med Vandmiljøplanens krav til fjernelse af organisk stof og næringssalte, var generelt gode til også at eliminere østrogen aktivitet og det samme gjaldt biologiske sandfiltre. Derimod var fjernelsen af østrogener i små traditionelle renseanlæg uden slambehandling ofte dårlig.

Selv om kortlægningen ikke pegede på, at østrogen aktivitet var et omfattende problem i ferskvandmiljøer i Danmark, var der dog en række emner og problemstillinger, der ikke blev belyst i tilstrækkelig grad til at konklusioner kunne drages. Det drejede sig f.eks. om drænvandsudledninger fra marker gødet med husdyrgødning eller slam, udløb fra ukloakerede enkeltejendomme samt effekter på vandkvaliteten af visse spildevandsudledninger til små vandløb, hvor spildevandstilførslen udgør en væsentlig del af den samlede vandføring. Det blev derfor besluttet at gennemføre en række supplerende undersøgelser for at få belyst disse emner nærmere.

Undersøgelsen

De supplerende undersøgelser af østrogen aktivitet i vandmiljøet har omfattet følgende tre hovedelementer:

- Kvalitet af vand og sediment i små vandløb opstrøms og nedstrøms spildevandsudløb fra traditionelle mekanisk-biologiske renseanlæg samt i selve udløbene fra disse anlæg. Der blev udvalgt 6 lokaliteter, der hver blev prøvetaget én gang.

- Indholdsniveauer i udløb fra septiktanke fra enkeltejendomme (eller små grupper af ejendomme) i det åbne land, der ofte udledes til vandløb via dræn eller andre ledninger uden yderligere rensning. Der blev taget 3-4 døgnprøver i løbet af en uge på hver af 5 udvalgte lokaliteter.

- Aktivitet i drænvand fra marker, der i foråret 2005 havde fået tilført enten husdyrgødning (gylle) eller spildevandsslam. Prøvetagningen blev gennemført på i alt 4 lokaliteter (tre gyllebehandlede marker og én slambehandlet mark) over en periode på 3 uger med start kort efter, at udbringningen havde fundet sted.

Alle udtagne prøver blev testet biologisk for samlet østrogen aktivitet, mens udvalgte prøver inden for alle kategorier desuden blev analyseret kemisk for indhold af steroidøstrogener.

Hovedkonklusioner

Ved de supplerende undersøgelser af vandløb med lille vandføring kunne der ikke påvises forøget østrogen aktivitet i vandprøver udtaget nedstrøms spildevandsudledninger fra traditionelle renseanlæg selv om udledningerne udgjorde 10-20 % af den samlede vandføring. Det skal dog bemærkes, at der på prøvetagningstidspunktet kun var signifikant østrogen aktivitet i to af de seks undersøgte udløb og ganske lav aktivitet i yderligere to. De kemiske analyser af nedstrøms sedimentprøver viste imidlertid, at der jævnligt må forekomme østrogen påvirkning idet der var en tydeligt forhøjet aktivitet fra steroidøstrogener i disse prøver.

Også ved sidste års kortlægning af østrogenaktiviteten i vandmiljøet blev der fundet ganske stor variation i niveauerne af østrogen aktivitet i udledninger fra traditionelle små renseanlæg, hvilket giver grund til at formode, at der generelt kan være betydelige forskelle i renseeffektiviteten inden for denne kategori.

De undersøgte udløb fra septiktanke ved enkeltejendomme udviste ligeledes betydelig variation i niveauerne af østrogen aktivitet, men i langt de fleste prøver var aktiviteten dog mindst 10-100 gange højere end de laveste kendte biologiske effektniveauer. Dette indebærer, at der er behov for en betydelig initialfortynding af septiktankudløb for at komme ned på uproblematiske niveauer, hvilket dog også i almindelighed skønnes at kunne opnås.

Den østrogene aktivitet i drænvand fra fire gylle- eller slamgødede marker (hhv. 3 og 1) bestemt ved bioassay blev i denne, supplerende undersøgelse fundet at være lavere end detektionsgrænsen i alle prøver på nær én, mens der ved kemisk analyse blev påvist ganske lave indhold af østrogener i 3 ud af 5 analyserede prøver. Ved kortlægningen i 2004 blev der fundet lidt højere, men stadig ret lave indhold af østrogener i de få undersøgte prøver. Det vurderes, at der skal en undersøgelse af længere varighed til for at kunne drage en endelig konklusion vedrørende risikoen for udvaskning af østrogener fra gylle/slambehandlede marker.

Projektresultater

Resultaterne af biologiske test og kemiske analyser af vand og sediment i små vandløb, der modtager renset spildevand fra små, traditionelle renseanlæg, er vist i tabel 1. Prøverne af udløbs- og vandløbsvand er udtaget som kvalificerede stikprøver (prøver sammenstukket af fem delprøver), og de nedstrøms prøver af både vand og sediment er udtaget i en afstand fra spildevandsudløbet, der omtrentligt svarer til 10 gange vandløbets bredde for at sikre en passende opblanding af spildvandet med vandløbsvandet.

Tabel 1
Østrogen aktivitet i udløb fra små renseanlæg og i de modtagende vandløb, hhv. i vandfasen og i sedimentet. Niveauerne er angivet som østradiol (E2) ækvivalenter (ng/L for vand og ng/kg tørvægt for sediment).

Lokalitet	Test eller analyse	Udløb	Vandløb, vand		Vandløb, sediment	
			opstrøms	nedstrøms	opstrøms	nedstrøms
W1	biologisk	i.p.	i.p.	i.p.	-	i.p.
	kemisk	-	-	<0.1	-	280
W2	biologisk	19.8	i.p.	i.p.	i.p.	i.p.
	kemisk	-	-	0.19	200	30
W3	biologisk	i.p.	i.p.	i.p.	i.p.	510
	kemisk	0.14	-	0.21	100	910
W4	biologisk	i.p.	0.37	i.p.	-	110
	kemisk	0.38	-	0.22	-	420
W5	biologisk	i.p.	i.p.	i.p.	-	i.p.
	kemisk	-	-	<0.1	-	190
W6	biologisk	3.94	0.36	i.p.	-	1160
	kemisk	-	-	0.10	-	810

i.p. = ikke påvist
- = ikke analyseret

Den østrogene aktivitet i afløbsvand fra septiktanke fra enkeltejendomme (eller små gruppe af ejendomme) i det åbne land blev undersøgt på fem lokaliteter, hvor der på hver blev udtaget døgnprøver gennem tre døgn i løbet af en uge (fire prøver på én lokalitet). Hovedresultaterne fremgår af tabel 2. Der blev kun påvist indhold af p-pille østrogenet i afløbet fra én septiktank. Her svarede niveauet til ca. 10 ng E2/liter.

Tabel 2
Østrogen aktivitet i afløbsvand fra septiktanke.
Resultater angivet i østradiol (E2) ækvivalenter (ng/L).

Septic tank	Biological testing		Chemical analysis of highest value in biological test
	Average	Range	
S1	88.2	55.1 - 125	84.1
S2	425	352 - 425	417
S3	28.5	24.5 - 31.5	12.2
S4	44.2	29.5 - 68.9	17.4
S5	4.93	neg - 14.8	4.16

Prøverne af drænvand fra gylle- og slamgødede marker (tre gyllegødede marker og én slamgødet mark) blev udtaget i maj måned 2005 som 2-3 eller 3-4 døgnsprøver over en periode på 3 uger på hver lokalitet (én lokalitet dog kun prøvetaget gennem én uge).

Kun i en enkelt prøve ud af i alt 20 kunne der påvises svag østrogen aktivitet (0,1 ng E2/liter) ved den biologiske test, mens der i tre ud af fem prøver, hvor der blev foretaget kemisk analyse, blev fundet indhold af steroidøstrogener. Niveauerne var dog meget lave; fra 0,10 til 0,32 ng E2/liter.

1 Introduction

1.1 Background

Within the last decade a number of international studies have described impacts on fish and other aquatic species resulting from exposure to endocrine disrupting chemical substances (in particular substances with estrogenic activity), and supporting preliminary findings of feminisation of fish in a Danish field survey was reported in 2000. The situation raised concern among scientists, politicians and the general public leading to a number of activities and projects aimed to further elucidate the issue including the Danish Environmental Protection Agency's (DEPA) launching of a large study named "Survey of Estrogenic Activity in the Danish Aquatic Environment" in late 2003 (Part A of the current study, published by DEPA as Environmental Project No. 977, 2005).

The survey had the objectives partly to provide data on the state of the Danish aquatic environment with respect to estrogenic activity and partly to identify the likely significant sources of contamination with estrogenic substances. Sampling was carried out at about 150 locations including various categories of streams/rivers and lakes, different types of wastewater treatment plants (WWTPs), drains from manure and sludge amended fields, septic tank effluents, urban rain runoff and fish farm effluents. The almost 350 samples were tested by the genetically modified yeast cell assay called "YES" and about 40% of the samples were additionally analysed chemically for their content of natural and synthetic steroid hormones (E1, E2, α-E2, EE2).

The results of the survey indicated that there was no widespread impact from estrogens in the Danish freshwater environment. However, downstream discharges of poorly treated wastewater to small receiving water courses, or in case of overflow episodes, the resulting environmental concentration would probably exceed effect levels known from the scientific literature.

In 33% of the samples from the aquatic environment the estrogenic activity if present was below the limit of detection. However, low estrogenic activity is found with the YES assay in almost all types of freshwater environments in Denmark, but typically at concentrations lower than 1 ng/L. In almost 70% of the water courses receiving WWTP effluents the level of estrogenic activity immediately downstream the discharge point was higher than the upstream activity. Further downstream of the discharge point the estrogenic activity had decreased to pre-discharge level.

However, due to various circumstances some sub-issues were not elucidated in the survey to the extent necessary for drawing reasonably firm conclusions regarding their possible environmental significance. This was in particular the case for manure or sludge affected field drains, septic tank effluents and the water and sediment quality in small streams receiving discharges from STPs with low treatment efficiency.

Therefore, it was decided that Part B of the survey (the current supplementary investigations) should focus on these issues.

Finally, a general observation in Part A was that most of the estrogenic activity in the different samples was due to the estrogens in their free form (without conjugation) whereas the conjugated forms only accounted for a minor fraction of the potential total activity. Therefore, it was decided to test and analyse only the free forms of the estrogens in Part B of the survey.

1.2 Objectives

The objectives of the supplementary investigation (Estrogen Survey, Part B) can be described as follows:

- to study further the significance of estrogen pollution sources in the open land such as drains from fields amended with animal manure or sewage sludge, and sewage from isolated (farm) houses i.e. septic tank effluents, and

- to assess the water and sediment quality in small streams receiving discharges from WWTPs with low efficiency in relation to elimination of estrogenic activity (called category C and D WWTPs in Part A of the survey i.e. plants with only mechanical or mechanical/biological treatment processes).

Finally, a few confirmatory tests of new samples from Part A locations were requested by DEPA and were included in the study.

1.3 Project implementation

Part B of the Survey of Estrogenic Activity in the Danish Aquatic Environment was implemented by the same project consortium that carried out Part A of the study:

COWI A/S - Project management, study design, planning and reporting, sampling:
Jesper Kjølholt (project manager from July 2005)
Frank Stuer-Lauridsen (project manager until July 2005)
Linda Høibye
Brian Ahlers.

Danish University of Pharmaceutical Sciences (DFU) - chemical analyses:
Bent Halling-Sørensen
Flemming Ingerslev
Kamilla Bach
Martin Hansen.

Eurofins Denmark A/S - sampling and sample pre-treatment:
Nis Hansen
Benny Køppen.

University of Southern Denmark (SDU) - biological testing:
Poul Bjerregaard
Bente Frost.

The supplementary investigations (including reporting) of the estrogen survey, which were carried out from March to November 2005, were steered by a Committee with the following members:

Jørgen Larsen, Danish Environmental Protection Agency (Chairman)
Lis Morthorst Munk, Danish Environmental Protection Agency
Helle Katrine Andersen, DANVA
Anne Marie Vinggaard, Danish Institute for Foood and Veterinary Research
Christian A. Jensen, Association of Danish Regional Authorities (Counties)
Steen Kristensen, Danish Medicines Agency.

1.4 Part B vs. Part A reporting

The investigations presented in this report are closely linked to the investigations carried out as Part A of the survey of estrogenic activity in the Danish aquatic environment. This was published in 2005 as Environmental Project No. 977: "Survey of Estrogenic Activity in the Danish Aquatic Environment" by the Danish Environmental Protection Agency (DEPA 2005).

As the Part B activities cannot be fully understood without bearing the design, methodologies and results of Part A in mind, this report has not been prepared as a full stand-alone report but rather an addendum to the above report and referring to it (as "Part A") for further details, where relevant.

2 Study design and methodology

2.1 Overview of investigations

To meet the overall study objectives, the following categories of estrogen pollution sources and aquatic environments were sampled and tested biologically with the YES assay and/or analysed chemically for contents of the steroid estrogens estrone (E1), 17ß-estradiol (E2), 17α-estradiol (α-E2), and 17α-ethynylestradiol (EE2):

- Effluents from selected category C/D WWTPs discharging into small streams (category C/D plants are simple, mostly small, WWTPs with only mechanical or mechanical/biological treatment of the sewage, cf. Part A section 2.2.1).

- The small streams receiving the discharged effluents from the above WWTPs. Sampling of stream water was carried out at one upstream and two downstream sampling positions (1 and 2) (cf. Part A section 2.2.3). Sediment sampling was in most cases only done at one downstream position (downstream 1).

- Septic tank effluents from isolated houses in the countryside.

- Drains from fields amended with either liquid manure (preferably pig manure) or sewage sludge.

- Confirmatory testing/analysis of a few samples from locations visited in Part A of the study.

More details on the considerations behind the sampling strategy and selection of locations are given in the following sections.

2.2 Sampling strategy and locations

Part A of "Survey of Estrogenic Activity in the Danish Aquatic Environment" was a broad study for which a large number of locations were selected. The aim was to describe the range of the "typical" or "average" situation both with regard to the aquatic environment and with regard to pollution sources, and not to focus on the "worst case" scenarios.

As the conclusion turned out to be that in general the estrogenic activity levels found in the investigated streams, rivers and lakes were relatively low compared to known effect levels, it was decided to focus more on the critical situations in Part B of the study.

Further, Part A of the study provided within certain categories, e.g. septic tank effluents and field drains, too few results to allow firm conclusions to be drawn. Therefore, Part B should also include such sample types to enable a better assessment of their potential importance in relation to contamination of the aquatic environment with steroid estrogens.

Specific comments on each sample category are given below.

2.2.1 WWTPs and receiving streams

The total of six WWTPs to be selected for Part B should all belong to either category C (mechanical-biological treatment) or category D (mechanical treatment) defined in Part A of the survey as these plants generally demonstrated low ability to remove steroid estrogens from the wastewater prior to discharge.

However, in Part A the effluents from C/D-plants only had a low or moderate impact on receiving stream water quality, probably due to the normally relatively high stream flow in comparison to WWTP effluent volume. Therefore, it was decided to select a new set of C/D-WWTPs for Part B having more critical ratios between effluent volume and stream flow i.e. ideally rather big C/D-plants with very small receiving streams (preferably max. ratio effluent/stream flow of 1:10). Ideally, the discharge should take place continuously. In reality, these requirements limited the possible WWTPs to category C as most category D plants would be too small and/or operating (discharging) too infrequently.

Further, the sampling should take place in the periods when the stream flow was as close to base flow as possible within the timeframe available. On the other hand, sampling of stream water should be also possible throughout the year, i.e. the streams must not dry out during summer, and sampling should be possible upstream the discharge point as well as downstream (up to a distance of at least up to 100 times the stream width). By this requirement a significant number of locations had to be given up as it is very common that small streams/ditches are covered and pipelined in some sections of their course.

Specific locations were selected based on a review of the county-wise listings of existing C/D WWTPs and subsequent personal contact to the responsible departments in the county administrations to obtain more detailed information about the status of the WWTPs and their receiving streams. In many cases further follow-up by contact to technical/WWTP administrations at municipality level turned out to be necessary to obtain the required updated baseline information.

2.2.2 Septic tank effluents

Suitable locations for sampling of septic tank effluents from isolated houses or farms in the countryside turned out in Part A of the survey to be difficult to find. Hence, this type of potential source of estrogen contamination was not fully examined in Part A and it was decided instead to further explore the issue in Part B as a significant number of such potential point sources exist in the open land in Denmark.

The composition of domestic sewage will differ from one household to another depending on the structure of the families (including sex and age). On the other hand, no significant regional differences in the domestic sewage composition were anticipated. Therefore, for practical reasons the identification of specific locations was largely restricted to an area in North Zealand where the consultant had long working experience within the sector including collaboration with the local authorities together with whom the specific locations were then selected.

One location in Jutland from Part A of the survey was, however, re-selected for the sampling at the very outlet from the tank while the other samples were taken in inspection wells at a relatively short distance from the tanks (sampling directly in the outlet would have required re-configuration of the system). Only tanks discharging into tight pipelines and not into porous field drains were selected for sampling to avoid possible drying out during the summer period.

2.2.3 Field drains

Planning of sampling in field drains within a limited time period is extremely difficult since the flow in the drains depend not only on the local soil and hydro-geological conditions but also strongly on the actual weather conditions, in particular the frequency, intensity and duration of rain episodes.

Field drains of relevance for this study should largely be rain-fed to enable detection of steroid estrogens percolating through the topsoil and into the drains. However, such drains are likely to dry out already during the spring and usually flow does then not re-appear until late in the autumn. On the other hand manure or sludge cannot be applied before the fields are dry enough to allow traffic with heavy vehicles. Therefore, in a study like this with just a few months available for the field activities, only a narrow "window" is open for obtaining the required samples.

Originally, it was planned to sample for a relatively short period at four locations for each type of waste material (manure and sludge). However, following consultations with the Geological Survey of Denmark and Greenland (GEUS), and learning from their experience with operating field drain sampling stations established with the aim to develop an early warning system for pesticide leaching (VAP), it was decided rather to select only two locations of each type and monitor each of them for a somewhat longer period (3 weeks). The monitoring should start shortly after application of the manure/sludge in order to catch possible "first flush" events caused by leaching through different types of macropores.

The drains for sampling from manure amended field were selected in collaboration with the counties responsible for the environmental monitoring in the so-called "LOOP" catchment areas (a number of particularly well described small catchments under the national water environment surveillance programme), as it was considered important to work in areas with well described physical environments in this particular part of the study. Unfortunately, it was not possible to locate sewage sludge amended fields in any of the LOOP areas, and therefore contact was made to a company distributing sewage sludge to farmers who suggested possible locations. Based on farmer interviews a presumably suitable location for sampling was selected.

2.2.4 Follow-up on Part A samples

In Part A of the survey relatively high levels of estrogenic activity (up to 6.2 ng E2 equiv./L) were observed in a forest lake selected as reference lake (i.e. no known contamination sources). A confirmatory biological test of the stored "bank" samples as well as a renewed spot sampling at the location was performed upon request from DEPA. One bank sample was also analysed chemically.

Likewise, DEPA requested a confirmatory analysis of the bank sample plus renewed (spot) sampling and testing at a field drain location (sludge amended field) which was found in Part A to exhibit inexplicably high estrogen activity (up to 36 ng E2-equiv./L).

Finally, DEPA requested three sediment samples (one upstream and two downstream) to be taken in receiving streams from category C WWTPs at two locations. It was agreed with DEPA that these two locations could be selected among the locations to be identified for the sampling programme for WWTP effluents and receiving waters described in Section 2.2.1.

2.3 Sampling programme and methodology

The strategy for and process of selecting the different sampling locations was described in the preceding section and in
Figure 2-1 the locations of the individual stations within each category are shown. The sampling programme can be briefly described as follows:

- Spot sampling of **WWTP effluents** from six category C wastewater treatment plants with a size corresponding to at least 100 PE (located in Hjørring, Arden, Tjele, Demstrup, Juelsminde and Stubbekøbing municipalities, respectively).

- Spot sampling of **stream water** in the streams receiving the treated effluents from the above WWTPs. Samples were taken at one upstream and two downstream stations (approx. 10 x stream width = downstream 1; and approx. 100 x stream width = downstream 2).

- **Sediment** sampling at the same locations; however upstream + downstream 1 + downstream 2 only in Arden and Tjele, while at the other locations sediment was only sampled at the downstream 1 position (actually the samples were taken at the point nearest to the downstream position with sedimentation conditions).
  The sediment samples were composite samples consisting of three sub-samples of the upper 2 cm of the sediment taken at a distance of 30-50 cm from each other. Different sampling equipment was used depending on the local conditions at each site.

- Time-proportional sampling of **septic tank** effluents at five locations of which four were in Karlebo municipality while the fifth was situated in Egtved. The latter was used for sampling directly at the outlet from the tank while at the others samples were taken in inspection wells at some distance (from a few metres up to about 100 metres) from the tanks. All samples were taken over 24 hours using automatic sampling equipment (time-proportional as flow-proportional equipment could not easily be installed). In Karlebo, samples were taken three days in a row while in Egtved four successive days of sampling took place.

- Time-proportional sampling of water from field drains dewatering a total of four fields amended with either liquid manure or sewage sludge. Each location except one was sampled for a period of 3 weeks with two sampling periods of 3-4 days each per week, i.e. a total of 6 samples per location. One location was erroneously only sampled for one week (i.e. two samples).
  Further, due to rapidly changing information and situations at a critical moment, three of the locations ended up being manure amended fields (two cattle manure and one pig manure - sampling of one of the cattle manure fields was disrupted after one week) while only one was a sludge amended field.

- Renewed spot sampling of Part A reference lake water at Sorte Sø, County of Funen, and of Part A sludge field drain at Blommenslyst, County of Funen.

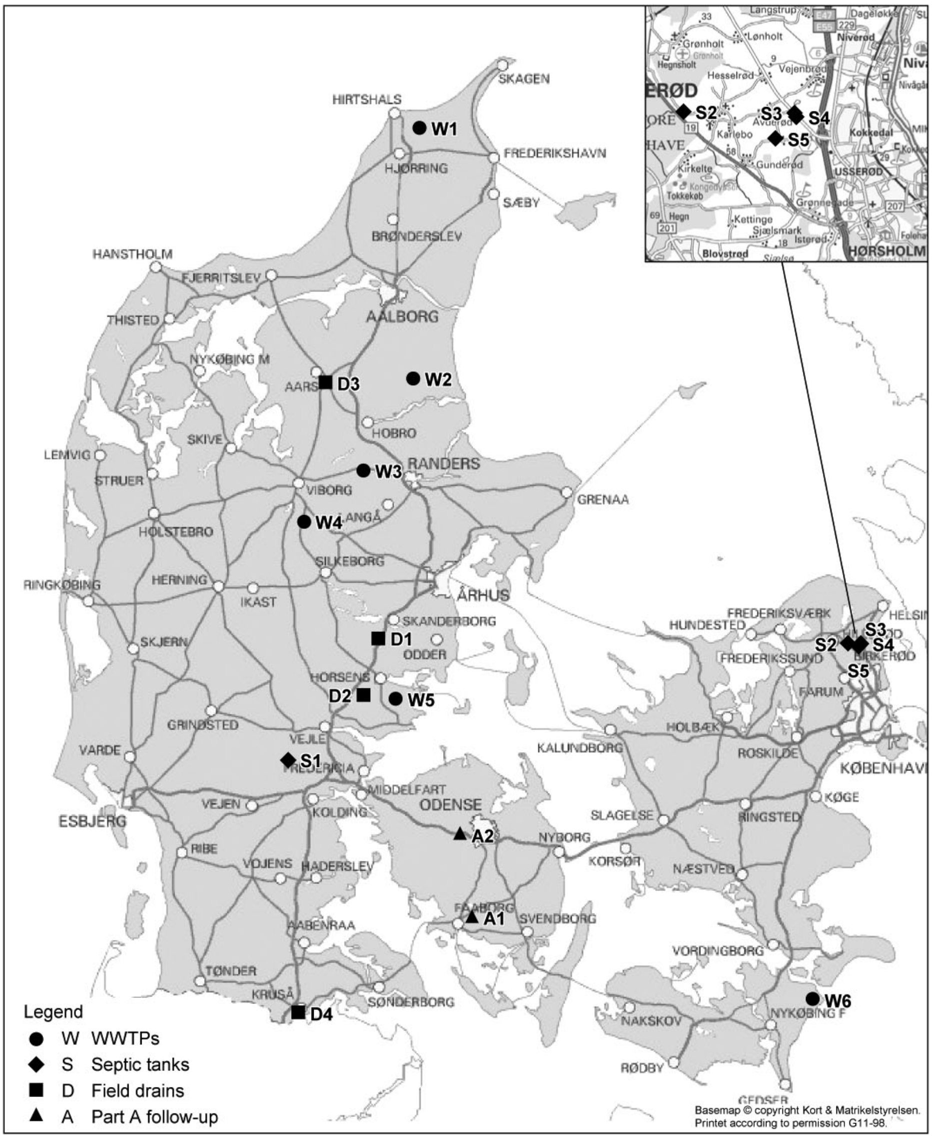

Figure 2-1 Location of sampling sites in Part B of the survey of estrogenic activity in the Danish aquatic environment.

The spot samples were taken by the procedure known as "qualified spot sampling" (combined sample of five sub-samples) as described in part A section 3.1.

For description of on-site conservation, storage and pre-treatment of aqueous samples, please see Part A sections 3.1 and 3.2.1. The sediment samples were not preserved on-site but were frozen immediately upon arrival to the laboratory and stored at -20 °C until work-up and subsequent testing/analysis.

An overview of the number of samples taken within each category as well as the associated numbers of biological tests and chemical analyses is given in Table 2-1 below.

Table 2-1 Overview of programme for sampling of the different sample categories and the associated biological testing and chemical analysis.

Sample type	Number of samples		
	Total*	Biological testing	Chemical analysis
WWTP effluents	6 x 1	6	2
WWTP receiving streams	6 x 3	18	6
WWTP receiving stream sediments	4 x 1 + 2 x 3	10	10
Septic tank effluents	4 x 3 + 1 x 4	16	5
Field drains, manure	2 x 6 + 1 x 2	14	4
Field drains, sludge	1 x 6	6	1
Other sample types	6	6	1
Total	76	76	29

* = number of locations x number of samples per location.

The sampling was carried out in the following sequential order:

1. Sampling of water from field drains (both manure and sludge amended fields). This was partly to catch possible initial breakthrough after application and partly to minimise the risk of drying out of the drains before sampling could take place (late April - May 2005).

2. Renewed sampling at two locations from Part A of the survey, a field drain (sludge amended field) and a reference lake. sampled in the spring to resemble the sampling time in Part A the best possible (May 2005).

3. Septic tank effluent sampling. As the samples were taken in tight pipeline systems this sampling would be less vulnerable to drying out in the summer than effluents being discharged into field drains. Samples had on the other hand to be taken before the start of the main summer vacation period (mid-late June 2005).

4. Sampling of WWTP effluents and water and sediments in receiving streams. This was planned to take place when the flow in the streams was close to base flow, normally in July-August. Sampling had, for capacity reasons, to be carried out in two steps (mid July and mid August).

2.4 Biological testing and chemical analysis

In Part A both the estrogenic activity from steroid estrogens in their free form (the active form) and the potential total activity of the free plus the conjugated forms (glucoronides and sulphates) were tested (by applying an enzymatic de-conjugation step in the sample pre-treatment to convert the conjugated estrogens into their free form).

It was, however, a general observation in Part A that most of the estrogenic activity in the different samples was due to the estrogens in their free form whereas the conjugated forms only accounted for a minor fraction of the potential total activity. This was observed both in the biological test and in the chemical analysis.

Therefore, it was decided to test and analyse only the free forms of the estrogens in Part B of the survey and, hence, the results to be presented in Chapter 3 of this report only regard the free estrogens.

2.4.1 Selection of samples for biological testing and chemical analysis

The number of samples within the different sample categories that were tested biologically and/or analysed chemically in Part B are stated in Table 2-1.

Sampling, testing and analysis of WWTP effluents were not included in the original programme. However, such testing/analysis was found to be highly relevant in order to enable interpretation of the results from the receiving stream samples, and was made possible as the number of field drain samples taken turned out to be lower than originally planned.

All samples were tested biologically while only selected samples were analysed chemically. Only in the case of sediments all samples were also analysed chemically. For all other samples the selection of samples for chemical analysis was not made until the results of the biological testing was available.

Selection of samples for chemical analysis:
WWTP effluents: Two locations showing no estrogenic activity in the biological assay were selected for chemical analysis to confirm this result.

WWTP receiving streams: The downstream 1 samples.

WWTP receiving stream sediments: All samples.

Septic tank effluents: The sample at each location exhibiting the highest estrogenic activity.

Field drains (manure + sludge): The first sample in each series + the single sample in which estrogenic activity above the detection limit was found (Løsning, week 2, 2. sample).

Part A samples: The renewed sample from the field drain at Blommenslyst.

2.4.2 Methodology - Aqueous samples

The methodologies and procedures applied in Part B for pre-treatment, biological testing and chemical analysis of aqueous samples were identical to the methodologies and procedures applied in Part A of the survey.

This means that the samples were cleaned up by solid phase extraction (SPE) using C_{18}-cartridges (+ silica gel for the sub-samples for chemical analysis), and subsequently tested biologically by the *in vitro* yeast cell assay known as the YES-assay and/or analysed chemically by GC-MS/MS after derivatisation with a mixture of MSDTFA, TMSI and DTE.

The methodologies and procedures are described more detailed in the Part A report, section 3.2 and appendices referred to in that section.

2.4.3 Methodology - Sediment samples

Testing and analysis of sediments were not included in Part A and therefore the applied method for sample preparation is described in detail below:

A sediment sample of approximately 30 g was dried at 25 ºC. For each analysis a sub-sample of approximately 5.0 g dried sediment was weighed out and mixed with 20 g Ottawa sand. Internal standards, deuteriated E1 and E2 (20 ng per sample), were added through the methanolic stock solution. The ASE-extraction cells for the pressurised liquid extraction (PLE) applied were lined with two cellulose filters in the bottom. Extraction was performed using methanol/acetone (1:1; v/v) and the following parameters: preheat (0 min); heat (5 min); static (5 min); flush (60%); purge (60 sec); cycles (2); pressure (500 psi); temperature (100°C).

The ASE extract was evaporated to dryness under a stream of nitrogen and reconstituted in 5 ml acetonitrile/MilliQ-water (2:3, v/v). The sample was then centrifuged at 3000 rpm for 3 minutes. The supernatant was transferred to a 250 ml blue-cap bottle, while another 5 ml acetonitrile/MilliQ-water (2:3, v/v) was added to the precipitate. The sample was mixed and centrifuged, and the supernatant again transferred to the same blue-cap bottle. The procedure was repeated once more. The combined extract in the blue-cap bottle was diluted with 150 ml MilliQ-water resulting in a MeCN content below 5%, which is required for the subsequent SPE (C18) procedure. pH was adjusted to pH =3.

The methodology for the subsequent biological testing and chemical analysis of sediment samples was identical to the one described above for the aqueous samples.

2.4.4 Detection and quantification limits

2.4.4.1 Biological assay

The detection limit in the YES assay was approximately 0.05 ng per litre in water samples and 100 to 200 ng per kg dry weight sediment (assuming that the absolute number of picogrammes (pg) estrogenic activity detectable are identical in the water and the sediment samples).

2.4.4.2 Chemical analysis

In part A an experimentally generated general LOD for the chemical analyses was determined to 0.1 ng E2 equiv./L for each component. In this determination, tap water was used instead of real samples (i.e. sewage effluent or surface waters) though, obviously, more analytical problems due to effects from the matrix could be expected if real samples were analysed. However, by comparing chromatograms from sewage effluent and surface water with those obtained with tap water, matrix effects generally appeared to have only minor importance and therefore the LOD in Part B is also assessed to be 0.1 ng E2 equiv./L. There were, however, situations where such problems occurred and resulted in elevated detection limits.

LOD for the sediment chemical analysis was determined to 25 ng/kg dry sediment. This was based on the same determination on tap water used as explained above. Also here chromatograms from sediment were compared with tap water and again matrix effects generally appeared to be negligible.

3 Results

3.1 WWTPs and receiving streams

The activities of free steroid estrogens in effluents, receiving stream waters and sediments were studied at six Category C (mechanical-biological treatment) WWTPs for which some basic data are presented in Table 3-1. All of the plants were constructed as closed tank systems which are believed to be based on (aerated) activated sludge processes though this has not in all cases been possible to verify.

The results (total activities) are shown in Table 3-2 while the chemical speciation of the total activity measured in selected stream water samples and sediment samples can be found in Table 3-3 and, respectively.

The sampling dates are also stated in Table 3-2. The weather conditions were dry at all locations immediately before and during sampling.

Table 3-1 General data about the selected WWTPs

WWTP	Cat.	Process*	PE	Estimated ratio, effluent:stream flow	Other remarks
W1. Mygdal	C	MB	180	(1 : 10)	High retention time
W2. Tisted	C	MB	100	1 : 5	-
W3. Tjele-Hammershøj	C	MBC	1670	(1 : 2)	High retention time
W4. Kjellerup-Demstrup	C	MBC	1100	1 : 6	Only domestic sewage
W5. Møgelkær	C	MB	1000	1 : 8	A prison is the main contributor (85% men)
W6. Moseby	C	MB	150	(1 : 5)	-

* M = Mechanical; B = Biological; C = Chemical precipitation.
( ) very uncertain estimate

Table 3-2 Activity of free estrogens in WWTP effluents, receiving streams and sediments. Levels in E2 equivalents (ng/L)

WWTP location	Sampling date (2005)	Test type	Effluent, E2-equiv. (ng/L)	Receiving stream, water E2 equivalents (ng/L)			Receiving stream, sediment, E2 equivalents (ng/kg dw)		
				up	down 1	down 2	up	down 1	down 2
W1. Mygdal	19.08	bio	neg	neg	neg	neg	-	neg	-
		chem	-	-	<0.1	-	-	280	-
W2. Tisted	19.08	bio	19.8	neg	neg	neg	neg	neg	1050
		chem	-	-	0.19	-	200	30	1740
W3. Tjele-Hammershøj	23.08	bio	neg	neg	neg	neg	neg	510	200
		chem	0.14	-	0.21	-	100	910	180
W4. Kjellerup-Demstrup	15.07	bio	neg	0.37	neg	0.87	-	110	-
		chem	0.38	-	0.22	-	-	420	-
W5. Møgelkær	14.07	bio	neg	neg	neg	neg	-	neg	-
		chem	-	-	<0.1	-	-	190	-
W6. Moseby	12.08	bio	3.94	0.36	neg	neg	-	1160	-
		chem	-	-	0.10	-	-	810	-

- = not analysed

Results from chemical analysis are presented as estrogen equivalents calculated as the sum of the concentrations of each of the analytes multiplied with conversion factors for the relative estrogenic activity of each analyte. The conversion factors were determined experimentally with the YES-assay by testing aqueous solutions of E1 (0.29) , EE2 (0.88) and α-E2 (0.04). Only values >LOD were included in the calculation.

As appears from Table 3-2 the estrogenic activity in the effluent samples was generally found to be low, below the detection limit (= neg) in four out of six samples tested biologically, and only the sample from Tisted exhibited an activity level of a magnitude corresponding reasonably to those found in Part A of the survey for the same WWTP category (C) (see section 6.1.2.1 in the Part A report).

In accordance with the effluent results also the levels in the water of the receiving streams are found to be low.

The results of the biological testing and the chemical determinations are found to correspond well to each other.

E1 was detected at low concentrations (i.e. < 1 ng/L) in all the effluent and stream samples that were analysed chemically. However, as the estrogenic activity of E1 is only 0.29 times that of E2, the calculated E2-equivalents were even lower and in two cases below the E2 quantification limit, LOQ, of 0.1 ng/L.

The highest total estrogenic activities among the "chemistry" samples were observed in the samples from Kjellerup-Demstrup WWTP and it receiving stream, Grundel Bæk. These were the only two samples in which the contraceptive agent EE2 appeared in concentrations above the LOQ.

Table 3-3 Chemical speciation of free estrogens in WWTP effluents and receiving streams (downstream 1 position).

WWTP location	Concentration (ng/L)				
	E1	E2	α-E2	EE2	E2-equiv.
Effluents					
W3. Tjele-Hammershøj	0.50	<0.1	<0.1	<0.1	0.14
W4. Kjellerup-Demstrup	0.44	<0.1	<0.1	0.28	0.38
Receiving streams (downstream 1)					
W1. Mygdal	0.22	<0.1	0.31	<0.1	<0.1
W2. Tisted	0.67	<0.1	<0.1	<0.1	0.19
W3. Tjele-Hammershøj	0.74	<0.1	<0.1	<0.1	0.21
W4. Kjellerup-Demstrup	0.45	<0.1	0.16	0.10	0.22
W5. Møgelkær	0.26	<0.1	0.33	<0.1	<0.1
W6. Moseby	0.33	<0.1	<0.1	<0.1	0.10

In the sediments (Table 3-4), the total estrogenic activity calculated as E2-equivalents was found by the chemical analysis to be in the range from 30 to 1740 ng/kg dw. E1 was found in all samples in concentrations ranging from 100 to 3180 ng/kg dw. E2 was found in 7 out of 10 samples in the range from 40 – 580 ng/kg dw. α-E2, that is related to farming animals, was only found in one sample at a level of 1540 ng/kg sediment.

In the sediment sample EE2 did, however, not appear in the sample from Kjellerup-Demstrup but was found instead in samples from two other locations.

Table 3-4 Chemical speciation of free estrogens in sediments from streams receiving WWTP effluents.

WWTP location	Sampling position	Concentrations (ng/kg dw)				
		E1	E2	α-E2	EE2	E2-equiv.
W1. Mygdal	down 1	120	40	1540	170	280
W2. Tisted	up	100	<25	<25	170	200
	down 1	110	<25	<25	<25	30
	down 2	3180	820	<25	<25	1740
W3. Tjele-Hammershøj	up	110	<25	<25	<25	100
	down 1	1590	60	<25	440	910
	down 2	500	40	<25	<25	180
W4. Kjellerup-Demstrup	down 1	440	290	<25	<25	420
W5. Møgelkær	down 1	390	80	<25	<25	190
W6. Moseby	down 1	790	580	<25	<25	810

It is noted that all sediment samples showed estrogenic activity according to the results of the chemical analysis and that the rather high levels observed (compared to the water samples) reflect well the lipofilicity of the un-conjugated steroid estrogens and the derived tendency to partition into (the organic fraction of) the sediment phase.

However, it was only possible to detect estrogenic activity in half of the sediment samples when applying the biological assay (Table 3-4). These samples were the same as those with the highest contents of estrogens in the chemical analysis.

3.2 Septic tank effluents

The results of the biological testing and chemical analysis of septic tank effluents stated as E2-equivalents appear from Table 3-5 while the composition of the individual steroid estrogen species in selected samples (those with highest activity) are shown in Table 3-6.

The samples from Egtved were taken at the outlet from the septic tank while the others were sampled in inspection wells of the pipelines transporting the effluents to the discharge or infiltration points. The distance from tank to inspection well ranged from a few metres and up to about 100 metres. All locations were sampled in the second half of June 2005.

Table 3-5 Free estrogenic activity in septic tank effluents.
Values in E2 equivalents (ng/L).

Septic tank location	Sample	Biological testing	Chemical analysis
S1. Egtved	day 1	125	84.1
	day 2	111	-
	day 3	61.7	-
	day 4	55.1	-
S2. Karlebo	day 1	425	-
	day 2	497	417
	day 3	352	-
S3. Fredensborg 1	day 1	31.5	12.2
	day 2	29.4	-
	day 3	24.5	-
S4. Fredensborg 2	day 1	29.5	-
	day 2	68.9	17.4
	day 3	34.2	-
S5. Gunderød	day 1	neg	-
	day 2	neg	-
	day 3	14.8	4.16

- = not analysed

The levels found in the effluents from Egtved are in line with the results from Part A, while the levels consistently observed in the samples from Karlebo are considered very high. In contrast to the Karlebo samples, the Gunderød samples demonstrate low, even negative, responses in the biological assay.

Four of the five locations have only one household connected to the septic tank system while at the last, Karlebo, seven households are connected. At Karlebo, the higher number of persons connected combined with the fact a woman living in one of the houses was pregnant at the time when sampling took place, may account for the high estrogenic activity observed at this location.

No explanation of the low or even absent estrogenic activity in the Gunderød samples can be given based on the information available about the location including the household.

Table 3-6 Chemical speciation of free estrogens in septic tank effluents

Septic tank location	Sample	Concentrations (ng/L)				
		E1	E2	α-E2	EE2	E2-equiv.
S1. Egtved	day 1	67.8	55.8	10.3	9.8	84.1
S2. Karlebo	day 2	447	287	68.9	<0.1	417
S3. Fredensborg 1	day 1	24.1	5.2	<0.1	<0.1	12.2
S4. Fredensborg 2	day 2	22.4	10.9	0.62	<0.1	17.4
S5. Gunderød	day 3	7.97	1.85	0.61	<0.1	4.16

The deviation between biological and chemical results appears to be somewhat bigger for the septic tank effluents than for the WWTP effluent and stream water samples. This could partly be due to presence of other chemical substances with estrogenic activities than the steroid estrogens (e.g. alkylphenols and phthalates) and partly be a matrix effect.

A relatively high level of the synthetic steroid estrogen EE2 (α-ethynylestradiol) was observed in the sample from Egtved while this substance was not detectable in any of the other samples. In Part A of the survey EE2 was only observed in very few samples.

3.3 Field drains

Sampling of drains dewatering fields amended with liquid manure was carried out at three locations, all located in Jutland. At one location (Ejer Bavnehøj), the sampling was by mistake discontinued after one week without replacement of the location by another.

The Ejer Bavnehøj and Odderbæk locations were fields amended with cattle manure while the Løsning field was amended with pig manure. A summary of data about the fields and the application of manure or sludge is presented in Table 3-7.

Table 3-7 Data on the sewage sludge and manure applied fields where drainage water was sampled. All locations area in Jutland.

Field location	Soil texture class	Waste type applied	Amount	Application date/period	Sampling period
D1. Ejer Bavnehøj	Sandy loam (JB6)	Liquid cattle manure	30 t ww/ha	week 14 + week 16	week 18
D2. Løsning	Sandy loam (JB5)	Liquid pig manure	30 t ww/ha	week 16-17	week 18-21
D3. Odderbæk	Not known	Liquid cattle manure	25 t ww/ha*	week 15-16	week 18-21
D4. Bov	Sandy loam (JB5)	Sewage sludge**	12 t ww/ha	10 April (end week 14)	week 18-20

* 25 tons liquid manure + 5 tons solid manure per hectare.
** Dry matter content: 16 %

In April when the application of manure and sludge took place (week 14 was 4-10 April and week 16 was 18-24 April) the weather was largely dry and it was not until around the turn of the month (April-May) that significant rain episodes occurred.

Heavy rains (12-34 mm) occurred throughout Jutland at the time when the first round of sampling was initiated. During the three weeks when sampling took place several smaller but no further major rain episodes were registered.

The estrogenic activities in the field drains (manure as well as sewage sludge) appear from Table 3-8. Only in one sub-sample detectable, very low estrogenic activity in the biological assay was found while in the others the response was negative.

Table 3-8 Free estrogenic activity in drainage water from fields amended with liquid manure or sewage sludge. Values in E2 equivalents (ng/L).

Field location	Sample	Biological testing	Chemical analysis
Fields amended with liquid manure			
D1. Ejer Bavnehøj	week 1, 1st	neg	0.32
	week 1, 2nd	neg	-
D2. Løsning	week 1, 1st	neg	0.10
	week 1, 2nd	neg	-
	week 2, 1st	neg	-
	week 2, 2nd	0.1	0.29
	week 3, 1st	neg	-
	week 3, 2nd	neg	-
D3. Odderbæk	week 1, 1st	neg	<0.1
	week 1, 2nd	neg	-
	week 2, 1st	neg	-
	week 2, 2nd	neg	-
	week 3, 1st	neg	-
	week 3, 2nd	neg	-
Fields amended with sewage sludge			
D4. Bov	week 1, 1st	neg	<0.1
	week 1, 2nd	neg	-
	week 2, 1st	lost*	-
	week 2, 2nd	neg	-
	week 3, 1st	neg	-
	week 3, 2nd	neg	-

* due to a perforation of the sampler tube caused by rodent "attack".

The results of the chemical analyses are in good accordance with the biological results although a low activity was observed by chemical analysis in two out of four samples that gave negative response in the biological assay. However, the maximum level of activity measured chemically was only 0.32 ng E2 equiv./L.

The composition of the chemically determined estrogenic activities is presented in substance)

Table 3-9. While the presence of α-E2 in the drainage water from Ejer Bavnehøj was expected as the manure applied on this field was from cattle, the presence of this steroid estrogen in the samples from Løsning is strange as the manure at this location should originate from pigs (which do not excrete much of this specific substance)

Table 3-9 Chemical speciation of free estrogens in drainage water from fields amended with liquid manure or sewage sludge

Field location	Sample	Concentrations (ng/L)				
		E1	E2	α-E2	EE2	E2-equiv.
Fields amended with liquid manure						
D1. Ejer Bavnehøj	week 1, 1st	0.40	<0.1	0.43	0.23	0.32
D2. Løsning	week 1, 1st	0.36	<0.1	0.32	<0.1	0.10
	week 2, 2nd	0.39	<0.1	0.30	0.21	0.29
D3. Odderbæk	week 1, 1st	0.27	<0.1	<0.1	<0.1	<0.1
Fields amended with sewage sludge						
D4. Bov	week 1, 1 st	<0.1	<0.1	<0.1	<0.1	<0.1

Further, it is noted that in the two samples with the highest activity, the major part of this is caused by the presence of EE2. This could indicate that the field drains may receive contributions from other sources than just domestic animals i.e. humans. Unfortunately, it has not been possible to obtain further any information that could lead to clarification of this question.

3.4 Part A follow-up

3.4.1 Reference lake

The reference lake, Sorte Sø (Funen), in which some estrogenic activity was observed in Part A in spite of the absence of agricultural or domestic contamination sources, was sampled again in May 2005 at exactly the same spot and using the same procedures as in Part A (2004). Further, two stored "bank" samples from Part A (sub-samples of the exactly the same samples from March and June 2004 that were analysed in Part A, see section 3.2) were tested with the YES assay. The results of the biological tests are shown in Table 3-10 while the chemical speciation of steroid estrogens in the June 2004 bank sample is shown in Table 3-11.

Table 3-10 Estrogenic activity in a new sample and re-tested bank samples from the reference lake Sorte Sø (location A1, fig. 2-1). Results of bank samples are compared to original 2004 data.

Sample	Estrogenic activity, ng E2 equivalents/L	
	2005 test	2004 test
New sample, May 2005	0.3	-
March 2004 sample	1.5*	1.5
June 2004 sample	12.3*	6.2

* Confirmatory test of stored 2004 sub-sample of the same sample tested in 2004.

Table 3-11 Chemical speciation of free estrogens in the June 2004 bank sample from Sorte Sø

Location	Sampling date	Concentrations (ng/L)				
		E1	E2	α-E2	EE2	E2-equiv.
Sorte Sø (ref. lake)	June 2004	10	0.25	0.14	<0.1	3.2

Thus, the high estrogenic activity observed in 2004 could not be confirmed by the new sample taken in May 2005. However, the two stored sub-samples of the 2004 sample still showed moderate and high activity, respectively, when re-tested with the YES assay and analysed chemically.

3.4.2 Drain from sludge amended field

A drain from a sludge amended field at Blommenslyst (County of Funen) demonstrated high estrogenic activity when tested with the YES assay in the 2004 survey (Part A) and was sampled again in May 2005. Also in this case two "bank" sub-samples from the same sample that was tested in 2004 were tested again. The results are shown in Table 3-12 and Table 3-13.

Table 3-11 Estrogenic activity determined by the YES assay in a new sample and re-tested bank samples from a sludge field drain at Blommenslyst, County of Funen (location A2, fig. 2-1). Results of bank samples are compared to original 2004 data.

Sample	Estrogenic activity, ng E2 equivalents/L	
	2005 test	2004 test
New sample, May 2005	97.7	-
May 2004 sample	5.0*	3.4
October 2004 sample	59.4*	36.1

* Confirmatory test of stored 2004 sub-sample of the same sample tested in 2004.

Table 3-12 Chemical speciation of free estrogens in drainage water from the field drain at Blommenslyst sampled in May 2005 (the field was amended with sewage sludge in the spring of 2004).

Field drain location	Sampling date	Concentrations (ng/L)				
		E1	E2	α-E2	EE2	E2-equiv.
Blommenslyst	May 2005	75.2	8.3	2.8	6.6	32.6

The observed high estrogenic activity levels are difficult to explain unless the drain also transports e.g. septic effluent. However, a recent inspection at the site by the local Water & Sanitation company has not provided any definitive information on the issue. A nearby farm has a septic tank but it is not obvious that the effluent could affect the field drain inspection well where the drainage water samples were taken.

Some difference between the biological and the chemical result is noted, which may be due to presence of other substances with estrogenic activity in the sample than the few steroid estrogens that were analysed chemically.

4 Discussion

4.1 Study design and methodology

4.1.1 Study design

Part B of the survey of estrogenic activity in the aquatic environment in Denmark was designed based on the results and experience obtained in Part A of the study as reported in Environmental Project 977 (DEPA, 2005).

However, while Part A was intended and designed to provide a broad impression of the "typical" situation with regard to both the state of the environment and the possible sources of contamination, Part B was initiated with the aim to focus specifically on two main aspects:

- investigate the state of vulnerable water bodies (i.e. small streams) receiving effluents from WWTPs with limited ability to remove steroid estrogens (category C and D plants cf. Part A, section 2.2.1), and

- further elucidate the level of estrogenic activity in two categories of contamination sources for which only insufficient data were obtained in Part A i.e. septic tank effluents and drainage water from field drains where manure or sludge had been applied.

4.1.1.1 WWTPs and small streams

Regarding the WWTP and associated streams, the main difference between Part A and Part B in the process of selecting the specific sampling locations was on the "stream side". While in Part A the WWTPs were selected with rather little emphasis on the size of the water body receiving the treated effluent it has been a central requirement in Part B that the effluent volume should be significant relative to the flow in the receiving stream.

In practice this implied that the search for suitable WWTPs was delimited to plants larger than 100 PE. At the same time it was attempted to find locations where the dilution of the effluent upon discharge into the stream would be less than 10 times of the effluent. Further, the sampling was carried out in July-August, which is the time of year when the stream flows are typically lowest.

The selection of sampling sites was carried out as a desk exercise using available GIS and internet information combined with interviews with local authorities and operational staff at the WWTPs or their municipalities. However, very little specific data about the stream flows was available.

Despite this, at five of the six selected locations it was estimated that the dilution was 5-8 times at the time of sampling and only at one (Mygdal) the dilution probably exceeded 10 times. Assuming estrogenic activity levels comparable to those observed in similar WWTP effluents in Part A, the activity level in the downstream water samples should be clearly above the quantification limit.

The WWTPs themselves were, similar to Part A, identified based on the information available in the counties' registrations and they are believed to be fairly representative of plants within the selected categories (C and D, though in reality only C was represented in the final selection of plants). However, there do exist, in particular in Category D, quite many WWTPs that are significantly smaller (fewer PE) than the selected ones and that also differ from these by not discharging continuously.

4.1.1.2 Septic tanks

It was assessed that the geographic variation in the composition of domestic sewage would be limited and therefore no attempt to distribute the required five locations for sampling of septic tank effluents over different regions. One suitable location in Jutland was already identified in Part A while the four others were identified within a small area in northern Zealand.

Four of the five locations have only one household connected to the septic tank system while at the last, Karlebo, seven households are connected. The latter represents a spectrum of households including one family in which there was a pregnancy at the time of sampling. The selected locations are, based on the information available, assessed to be representative of septic tank effluents nationwide.

4.1.1.3 Field drains

All the field drains locations are situated in the eastern half of Jutland, and all on soils with a moderate content of clay (10-15%; soil classes JB5-JB6), which is fairly typical of Danish agricultural soils. All the selected drains are largely rain-fed and normally run dry during summer.

To the extent possible the fields were selected within a number of small catchment areas, which under the national water and environment monitoring system, NOVANA, are designated for monitoring and evaluation of nutrient and contaminant releases to the freshwater environment. The three manure amended fields are situated in such an area while a suitable sludge amended field could only be found at another location.

4.1.2 Methodology

The sampling procedures and methodologies of biological testing and chemical analysis were identical to the ones applied in Part A. However, as sediments were not analysed in Part A the methodology applied for this purpose was new (within the framework of the study). Only free estrogens were tested/analysed in Part B because the conjugated forms were shown in Part A to account only for a minor part of the overall estrogenic activity. Further, in sediments conjugated estrogens are not expected to sorb to sediments due to their higher water solubility than the free estrogens.

The laboratory procedures and methodologies were tested and documented thoroughly in Part A of the survey (DEPA 2005), but also in Part B all work-up, tests and analyses of real samples were accompanied by blank samples and samples with standard addition of steroid estrogens to have a continuous control of possible background contamination and/or malfunction of the equipment and instruments.

As no problems with background contamination or lack of precision or sensitivity was observed when running the blank and standard samples the results of the real samples are considered to be valid.

4.2 WWTPs and receiving streams

4.2.1 Estrogenic activity in effluents and streams

The results of the biological testing and chemical analysis of WWTPs and associated stream samples including sediment are presented in chapter 3, Tables 3-2 and 3-3.

It appears from Table 3-1 that a significant biological estrogenic activity was observed in the effluent from Tisted WWTP (about 20 ng E2-equiv./L) while a somewhat lower activity was found in the effluent from Moseby (about 4 ng E2-equiv./L).

In the effluents at the four remaining WWTPs the activity was below the detection limit. However, by chemical analysis a very low activity could be observed in two of the effluents where the biological assay did not give any response.

The apparent lack of estrogenic activity was unexpected as in Part A the effluents from the same categories of WWTPs all showed significant activity (see Table 4-1, which summarises the WWTP effluent results in Part A). Only the result from Tisted is at a level that is comparable with those found for C/D plants in Part A.

It has only been possible to find a likely explanation of the lacking estrogenic activity at two of the WWTPs, Mygdal and Tjele-Hammershøj, at which we have been informed that the retention time is high (> 2 days).

There are no indications of problems with field or laboratory procedures and follow-up enquiries to the other WWTPs have not revealed any atypical conditions. All samplings took place in dry weather and, hence, dilution by rainwater cannot be an explanation either.

Table 4-1
Summary of tables 6-3 and 6.4 in Part A of the survey: Median and max. content of free estrogenic activity in the effluents from wastewater treatment plants in categories A-F. Measured biologically and chemically and presented as ng Estradiol equivalents/L. N, the number of samples, is separated into values <LOD, values >LOD, and all values.

Category	Type	Activity of free estrogens (E2 equiv., ng/L)					
		Biological assay			Chemical analysis		
		$n_{below/above/all}$	*median*	*max*	$n_{below/above/all}$	*median*	*max*
A	MBND(C)	5/10/15	2.8	30.4	2/6/8	1.3	15.5
B	MBN(C)	0/13/13	2.4	82.4	1/7/8	0.6	6.5
C	MB(C)	0/13/13	59.1	217.1	0/8/8	29.5	71.3
D	M(C)	0/16/16	21.8	167.5	0/13/13	9.0	123.5
E	Reed bed	2/9/11	8.2	17.8	1/7/8	3.0	5.8
F	Sand filter	6/3/9	0.2	17.5	6/4/10	0.4	2.15

In accordance with the lacking estrogenic activity in the effluents none or only little and sporadic activity could be detected in the stream samples - not even in Skibsted Å, the stream receiving the discharge from Tisted WWTP, although the effluent volume in this case was rather significant relative to the stream flow at the time of sampling (ratio roughly estimated to about 1 : 5).

In summary, the tests and analyses of stream water downstream the discharge points of small WWTPs have not, neither in Part A or B (results from a total of 18 category C/D plants), given any indication that such discharges significantly increase the estrogenic activity in the streams though a number of small increases (and one significant) were observed in Part A of the survey.

4.2.2 Estrogenic activity in sediment

All sediments demonstrated significant contents of steroid estrogens when analysed chemically and about half of the samples also gave a response in the biological assay. In the majority of samples wihout a biological response the chemically determined contents were below the detection limit of the YES assay (approximately 100 ng E2 equiv./kg dw).

The levels range from 30-1740 ng E2 equiv./kg dw in the chemical analysis and from <100-1160 ng E2 equiv./kg dw (7 out of 10 samples showed estrogenic activity) in the bioassay i.e. a significant enrichment compared to the levels in the stream water. The highest levels were found downstream of the Tisted WWTP discharge point and significant contents were also found in Lyremoseløbet, the stream receiving the effluent from Moseby WWTP.

These results demonstrate that despite the absence of activity in the water samples the WWTPs obviously release estrogens that, due to their lipofilicity (log P = approx. 3.5) when appearing on the free form, tend to sorb to particulates (organic matter) and precipitate in sedimentation sections of the streams. Further, the results indicate that enrichment of sediment with steroid estrogens occur downstream of category C treatment plants.

A comparison of the chemical and biological results shows that assessment of estrogenic activity in sediments only by use of a biological test in some cases may be insufficient due to poorer sensitivity of the latter. 30% of the samples showed no activity in the bioassay while activity was observed in all samples when analysed chemically.

The levels of estrogens found in Danish sediments (see Table 3-4) are comparable to or lower than levels found in other European countries. A Spanish study using chemical analysis showed that in river sediment from the Catalonia region much higher levels were found than in Denmark. E1 was found at the level of 11,900 ng/kg dw and EE2 at 22,400 ng/kg dw. In the present study (i.e. Part B) E1 was found at between 100 and 3,180 ng/kg dw and EE2 between 170 and 440 ng/l (3 out of 10 samples). Thus, the values found in Denmark are roughly a factor 10 lower than those from Spain

In Germany, levels similar to the Danish were found of E1 (up to 2,000 ng/kg dw) and EE2 (900 ng/kg dw) in samples of sediment from a polluted river, the River Rhine (Ternes et al. 2002).

The biological/ecological significance of such sediment levels is at present not possible to assess.

4.2.3 Possibilities of upgrading existing small WWTPs

For assessment of the possibilities of upgrading the existing small WWTPs for better removal rates of estrogenic substances, it is essential to 1) assess the mechanisms in wastewater treatment plants that influence the treatment performance and 2) specify the type and process configurations of the plants that shall be upgraded.

Based on previous investigations, it is assessed that the lower removal rates for estrogenic substances for small treatment plants (MB plants) compared to bigger (MBN and MBND plants) are associated with one or more of the following circumstances:

1. Low sludge age.
   Low sludge age in a wastewater is a disadvantage for slowly growing bacteria, which will be washed-out and hence it will not be possible to sustain some specific bacteria in the biological treatment units. This is a phenomenon that is very essential to bear in mind when designing a WWTP. For nitrifying treatment plants, for instance, it is essential to have a high sludge age, i.e. minimum 15-20 days for Danish conditions, in order maintain nitrifying bacteria in the plant. A similar mechanism might be relevant for removal of estrogenic substances, i.e. bacteria with high potential for decomposing estrogenic substances might be washed out of the biological treatment plant, if the sludge age is too low.

2. Low hydraulic retention time.
   Estrogenic substances can be regarded as moderately biodegradable organic matter, meaning that a certain minimum retention time in the process tank is necessary for a high degree of decomposition of the estrogenic substances. For small mechanical-biological treatment plants the hydraulic retention time in the process tank is typically around 4-8 hours, whereas average retention time in bigger MBND plants is close to one day (24 hours). Furthermore, the variation in incoming wastewater flow is bigger for small plants and therefore the retention time in peak hours is less.

3. Poor removal rate for suspended solids.
   Steroid estrogens in their free form tend, when discharged from wastewater treatment plants, to be attached to the suspended solids. A poor removal of suspended solids will hence have a direct influence on the removal rates for estrogens. Small treatment plants have, in general, poorer treatment results for suspended solids due to less monitoring and control systems and generally lower attention.

Low sludge age and low hydraulic retention time are, of course, to some degree linked together as both can be increased by construction of a bigger process tank at the treatment plant.

Most existing small WWTPs in Denmark of the category mechanical-biological plants (MB) are based on activated sludge technology, i.e. the treatment plants are constructed with the following process configuration:

- screens
- grit and grease chamber

- aerated activated sludge tank
- secondary clarifier
- sludge thickener

A minor part of the small treatment plants are based on fixed film technology, i.e. typically with a trickling filter still followed by a secondary clarifier.

Typically sludge is thickened at the treatment plant and transported to a central WWTP for mechanical dewatering. Some plants have, however, sludge drying beds for final dewatering.

Mechanical plants (M), typically very small treatment plant, are constructed as large septic tanks followed by direct discharge to the recipient or through an infiltration system. Sludge from the plants are emptied regularly (1-2 times per year) and transported to a central WWTP for further treatment.

Based on the above assessments of mechanisms that influence the treatment performance and the type and process configurations of the small treatment plants, the following improvement possibilities of the treatment performance in relation to enhanced removal of estrogenic substances can be proposed:

1. Increase sludge age by:
   - increase process tank volume
   - increase sludge concentration in the existing process tank, if this is possible in relation to the capacity of the secondary clarifier

2. Increase hydraulic retention time by:
   - increase process tank volume

3. Improve removal of suspended solids by:
   - improve monitoring and control of secondary clarifiers (turbidity meter, sludge blanket level meter)
   - improve performance of secondary clarifiers (installation of lamellas, Flockbee, etc.)
   - increase secondary clarifier volume
   - installation of polishing unit (conventional sand filter, biological sand filter, lagoons, etc.)

In general, most of the above suggestions demand a relatively high investment, i.e. typically 20-50% of construction costs for a new treatment plant. Upgrading of the treatment plant by increasing of the process tank volume can typically also improve the treatment performance for ammonia removal (i.e. upgrading to MBN-plant).

Beside the above mentioned improvements, it is expected that a general optimization of the treatment plant operation also will have an effect on the removal rate for estrogenic substances. These optimizations could include improved control of sludge concentration and levels in the different treatment units, improved oxygen control, etc. Measures for improved control and monitoring can, however, be implemented for a substantially lower investment.

4.3 Septic tanks

Significant estrogenic activity was found in septic tank effluents at four out of five investigated locations (see Table 3-4), while at the fifth location the effluent demonstrated none or only relatively low activity. The significant activities are in line with the findings at similar locations in Part A while the low activity at the Gunderød location remains inexplicable.

At one location very high activity levels were observed, about 350-500 ng E2 equiv./L, maybe caused by a pregnancy at the time of sampling in one of the seven families connected to the system. Only in one effluent was the synthetic steroid estrogen EE2 present at a level higher than the limit of detection, namely 9.8 ng/L. This result is in accordance with Part A where EE2 was also only rarely detected in the samples.

The results show, as expected, that the content of steroid estrogens typically remains high in the undiluted sewage after having passed through a septic tank. Hence, if discharged directly into a stream, this type of effluent will require significant dilution to reduce the estrogenic activity to a level below those that in experimental studies have been shown to cause biological effects.

Normally, a sufficient dilution is expected to take place as the effluent volume will be small compared to the flow in most streams. During summer effluents discharged into field drains may even dry out before reaching the stream and not be discharged until late in the autumn where the flow is higher and where the majority of the estrogens have probably undergone degradation by micro-organisms.

4.4 Field drains

The drainage water from two fields where liquid manure (pig and cattle, respectively) had been applied and one field where municipal sewage sludge had been applied was monitored during a period of three weeks in the spring of 2005, while water from a fourth drain at a cattle manure amended field was monitored for one week. The manure and sludge application took place in dry weather around mid April while the monitoring was not initiated until around 1st of May as no significant rain episodes, which could provoke estrogen leaching, occurred until then.

The biological testing revealed no estrogenic activity in the drainage water from any of the locations, except a very low activity (0.1 ng E2 equiv./L) in a single sample from the pig manure amended field.

Chemical analysis was carried out on the samples from the first sampling round at each location plus of the sample where activity was observed in the bioassay. In two of the four "round 1" samples a low content of steroid estrogens was found (0.32 and 0.10 ng E2 equiv./L) and the biological observation of activity in one sample was confirmed by the chemical analysis (0.29 ng E2 equiv./L). In the two samples with the highest activity (as E2 equiv.), the main contribution came from EE2, which was not expected to appear in the samples.

Thus, the quite short monitoring of field drains at a few fields having received either liquid manure or sewage sludge has not given any indication that high levels of estrogenic activity might appear in this possible source of aquatic contamination. In Part A low levels of estrogenic activity was observed in a minor part of such field drains while high levels (up to 36 ng E2 equiv./L) were observed in only one drain - from a sludge amended field at Blommenslyst, which therefore was re-investigated in Part B.

The re-investigation confirmed the previous results but has provided no conclusion as to the cause of this. It is, however, considered unlikely that such high levels can occur as a result of leaching of sludge contaminants through soil. In the specific case it is even more unlikely as the sewage sludge was applied more than one year before the re-sampling of the location took place.

4.5 Part A follow-up

The follow-up on specific Part A samples comprised renewed sampling and testing of a reference lake, Sorte Sø, and of a drain at a field where sewage sludge was applied in the spring of 2005; Blommenslyst. Comments on the latter were given above.

Sorte Sø is a slightly acidic forest lake with an apparently high content of dissolved humic substances. There are no visible anthropogenic sources of contamination of the lake but nevertheless the lake water exhibits a higher than background estrogenic activity.

A confirmatory chemical analysis of one of the bank samples of Sorte Sø water from 2004 (Part A) was included in Part B of the survey. The result showed that half of the total estrogenic activity observed in 2004 could be explained by the (unexpected) presence of steroid estrogens in the lake water and thereby that a hitherto unknown (and not immediately visible) anthropogenic contamination source may exist in the vicinity of the lake.

5 Conclusions

5.1 Simple WWTPs

There appears to be some discrepancy between the findings in Part A and Part B of the survey regarding the estrogenic activity in the effluents from Category C WWTPs. However, also in Part A there was a quite significant variation among the results within this category. It is, though, still the overall impression that the small mechanical and mechanical-biological WWTPs generally are not very effective with regard to elimination or reduction of steroid estrogens.

The number of category C plants in Denmark appears to be decreasing, from 336 in 2001 to 277 in 2004, while the number of category D plants has almost remained unchanged over the same period (DEPA 2002, 2003, 2004, 2005b).

The technical possibilities for improvement of the performance of the small mechanical and mechanical-biological treatment plants can typically include the following items in prioritised order:

1. General optimisation of treatment plant operation by improved monitoring and control sludge concentration in the process tank, oxygen supply and discharge of suspended solids.

2. Increase of sludge age and hydraulic retention time in the process tank. This could have the environmental side-effect that the plant is upgraded from MB plant to MBN plant.

3. Improve the performance of secondary clarifier by installation of lamella separators, Flockbee or similar or by construction of additional secondary clarifier volume.

4. Installation of polishing unit (conventional sand filter, biological sand filter or lagoons.

The best technical-economical solution for the individual treatment plants should be based on an individual assessment. Shutting down of the small treatment plant and pumping of wastewater to a central WWTP can for some plants be the best technical-economical solution.

5.2 Septic tank effluents

Septic tank effluents generally contain steroid estrogens at levels requiring 10-100 times dilution (or more) before being brought down to concentration levels below biological effect levels. More than half of the septic tanks in the open land in Denmark discharge into field drains from which only half of the volume is estimated to reach surface waters while the rest seeps out of the drains and into the soil where the estrogens will be degraded if/when aerobic conditions prevail.

Several thousands septic tank systems are in operation on properties in the Danish countryside. In DEPA's annual "Point source report" for 2004 (DEPA 2005b) the number of septic tanks discharging directly or via field drains is reported to be around 51.000 and 89.000, respectively (DEPA, 2005b). The majority of these, 41.000 and 66.000, respectively, are associated with single houses/farms while the remaining are properties in either villages or in recreational areas (summer cottages).

The majority of the municipalities had by the end of 2004 agreed on local sanitation plans, which should ensure improved treatment at 59% of the 96.000 properties for which improved treatment is required in the regional physical planning (DEPA 2005b). The majority of the plans are expected to be implemented before 2010 and, thus, the number of systems discharging directly or via drains into the freshwater environment is expected to decrease significantly in the coming years.

5.3 Field drains

Within the framework of this survey (Part A and B) it has only been possible to carry out a rather limited investigation of the possible contamination of the aquatic environment by discharges of water from field drains, which are widespread in many Danish agricultural areas. The duration of the monitoring was up to three weeks per location, but in reality continuous monitoring covering at least a full hydrological cycle (i.e. at least one year) is necessary before firm conclusions can be drawn. Further, a wider range of agricultural soil types should be studied.

The limited monitoring that was carried out in this study did, however, only reveal presence of low or undetectable estrogenic activity in drainage water from fields where liquid manure or sewage sludge was applied to the soil shortly prior to the sampling.

Further, the monitoring of surface water from streams and small lakes in husbandry areas in Part A of the survey showed that the levels were mostly below or around 1 ng E2 equiv./L and only in one case did the level in a stream exceed 5 ng E2 equiv./L. The latter result could not be confirmed by the chemical analysis of the same sample.

5.4 Impact of discharges on small streams

5.4.1 Impact on water quality

The survey (Part A and B) has demonstrated that low levels of estrogenic activity occur in streams where discharge of treated effluents from Cat. C/D WWTPs take place. However, only at one out of a total of 18 WWTPs where up- and downstream samples were taken did the activity level increase significantly as a result of the effluent discharge. In all other cases the level remained within the "normal" range observed in streams i.e. from the sub-ng E2 equiv./L level to the 3 ng E2 equiv./L level.

Thus, it has not in this study been possible to demonstrate that even significant estrogenic activity levels in sewage effluents should generally lead to levels in the freshwater environment where significant biological effects are known to occur.

However, also other sources such as field drains and septic tank effluents contribute to the overall load on the small streams in arable areas. The largest volume of water from these two types of contamination sources stems from the discharges of drainage water from fields. In some parts of Denmark a significant fraction (even the majority) of the fields are fertilised with pig or cattle manure while the application of sewage sludge only takes place on a rather limited fraction of the total agricultural area in Denmark. However, only low (sub-ng) levels or even no estrogenic activity could be detected in field drain water samples in this investigation.

In contrast to this, septic tank effluents generally show significant estrogenic activity i.e. levels some 10-100 times (or more) higher than levels where biological effects have been observed. The volume of these effluents reaching the streams will, however, in most cases be small compared to the flow in the streams though the existence of local "hot spots" is possible.

In the survey of streams and rivers of varying size in Part A (and the few samples in Part B) of the survey, the levels of estrogenic activity was generally at the sub-ng - 1 ng E2 equiv./L and did only in a few cases exceed a level of 3 ng E2 equiv./L. In England, the NOEC of estrogenic activity is presently considered to be 1 ng E2 equiv./L but this value may be subject to revision.

5.4.2 Impact on sediment quality

All sediments demonstrated significant contents of steroid estrogens when analysed chemically and about half of the samples also gave a response in the biological assay. The results demonstrate that despite the absence of activity in the water samples, the WWTPs obviously release estrogens that, due to their lipofilicity when appearing on the free form, tend to sorb to particulates (organic matter) and precipitate in sedimentation sections of the streams.

The analyses in Part B indicate that enrichment of sediment with steroid estrogens occur downstream of Category C treatment plants. The levels of estrogens found in Danish sediments are comparable to or lower than levels found in other European countries.

5.5 Background levels

The background or reference level of estrogenic activity in streams was, when studied in Part A, almost consistently under the limit of quantification (only 1 out of 11 barely above the LOQ).

The background level in lakes was somewhat higher, with a median level of 0.7 ng E2 equiv./L. In one of the reference lakes, Sorte Sø, the activity was about 6 ng/L and therefore this lake was re-investigated in Part B. The analysis of the stored samples from 2004 confirmed the high activity found last year while a new sample taken in May 2005 only showed little activity.

A chemical analysis of a bank sample of lake water showed that half of the total estrogenic activity observed in 2004 could be explained by the presence of steroid estrogens. This indicates that a hitherto unknown (and not immediately visible) anthropogenic contamination source may exist in the vicinity of the lake.

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