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Estrogens in Swiss Rivers and Effluents – Sampling Matters

Etiënne L. M. Vermeirssen*, Rik I. L. Eggen, Beate I. Escher, and Marc J.-F. Suter

Abstract: Estrogenic activity of treated sewage effluents and receiving waters surfaced as an issue of general concern some 15 years ago. Since then, a large number of studies investigated the distribution and nature of the estrogenic substances in various aquatic ecosystems. Within NRP50, a five-year Swiss research programme on endocrine disruptors, four field studies were performed to characterize the presence of environmental estrogens in rivers and effluents. The estrogenic activity was quantified with a biological assay – the yeast estrogen screen. An overview of the sampling approaches and results of the various studies is presented here. In a first study, using grab sampling, it appeared that estrogenic activity in river water was highly variable. Average estrogenicity values did not correlate with sewage treatment works and/or river characteristics, *e.g.* effluent dilution factor. However, variability was not 'random' but clearly associated with river size, and possibly its discharge. A second study specifically addressed this issue of variability of estrogenicity. The study was conducted at a single effluent source and its receiving river. Variability of estrogenicity in the grab samples was again large but again not 'random'; some of the variability was explained by the time over which the effluent resided in the treatment process. In a third study it was explored if passive sampling would be a better way to assess average estrogenicity. Indeed, passive samplers identified sources of estrogens, and passive sampling data correlated well with both repeated grab sampling and bioaccumulation data. Subsequently, passive samplers were deployed across Switzerland in a fourth study. It involved 22 effluent discharges and the associated rivers. Data analysis of the last study is still ongoing, preliminary observations are discussed here. Although a lot could be learned from repeated grab sampling campaigns, it emerged that passive sampling is a very effective and appropriate technique to assess: i) effluent treatment efficiency, and ii) the chemical load of river water. For these reasons it is a valuable monitoring tool for water quality criteria assessments as well as for studies that aim to link exposure and effect. The passive samplers that are currently available not only target estrogenic substances, but many other polar organic compounds of concern, such as antibiotics (see NRP49), other pharmaceuticals and biocides.

Keywords: Endocrine disruption · Estrogenic activity · Passive sampling · River water · Treated sewage effluent

Introduction

A study published in 1994 by Purdom and co-workers from the United Kingdom[1] highlighted that treated sewage effluents contain substances that are estrogenic to fish. Given the fact that many rivers receive such effluent, the observation pointed to a probable general exposure of aquatic wildlife. As river water is a widely used source for drinking water, and some rivers carry a very high load of effluent, human exposure was not hypothetical and helped raise the profile of the study.

Later it was established that the exposure of fish is indeed widespread[2] and a worldwide phenomenon.[3] At around the same time it emerged that natural steroids, notably estrone, are the main estrogenic component in treated domestic effluent.[4] The potent synthetic estrogen 17α-ethinylestradiol has also occasionally been identified in effluent and river water. However, besides the steroidal estrogens, domestic effluent contains many industrial chemicals that possess estrogenic properties: *e.g.* phenolic compounds such as nonylphenol and bisphenol A[5] (NRP50 project PHENCON), flame retardants (projects FLARE and ENDAIR[6]) and UV filters[7] (project HAUS). Beside household and industrial sources, also agriculture contributes to the pool of endocrine disrupting chemicals, including steroidal estrogens[8] but also phytoestrogens and estrogenic mycotoxins have been considered.[9]

Although various studies indicate that the steroidal estrogens are the main causative agents of the estrogenic activity of effluent and receiving waters, there is a risk associated with a targeted analysis for just a limited number of known estrogenic substances. On the other hand, there is a great cost associated with a much broader chemical screening that may still miss unknown estrogens. These issues can be partially circumvented by employing biological assays that are based on an estrogenic mode of action. Various such assay systems have been developed (*e.g.* E-

*Correspondence: Dr. E. L. M. Vermeirssen
Eawag Swiss Federal Institute Aquatic Science and Technology
Department of Environmental Toxicology
Überlandstrasse 133
P.O. Box 611
CH-8600 Dübendorf
Tel.: +41 44 823 5329
Fax: +41 44 823 5547
E-mail: etienne.vermeirssen@eawag.ch

Screen;[10] yeast estrogen screen, YES[11] and ER-CALUX[12]). Using combinations of chemical analysis and bioassays, many countries initiated large national surveys to address the issue of estrogens in the aquatic ecosystem.[13,14]

At the start of the NRP50, a five-year Swiss research programme on endocrine disruptors, some background information was already available on individual estrogenic substances in the Swiss aquatic ecosystem, particularly on nonylphenol.[15] Furthermore, various effluent and river water samples had been tested in the YES in combination with chemical analysis of target compounds.[16] Within NRP50 these efforts were continued with four studies that targeted the characterization of the estrogenic activity in Swiss rivers and effluent. The results of these studies are collated here, together with a comprehensive overview of the sampling strategies.

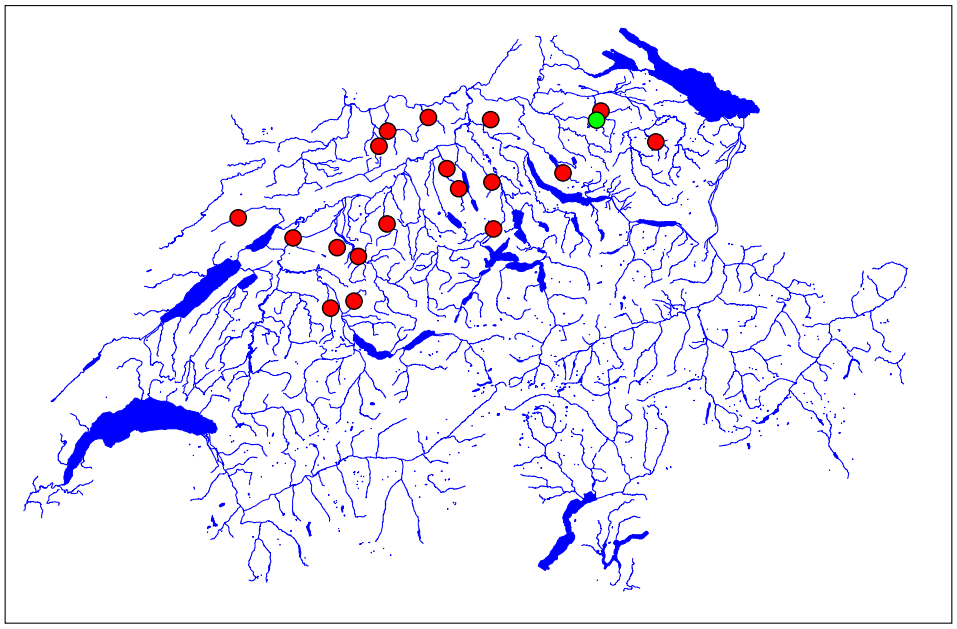

Fig. 1. Red circles on the map of Switzerland (© SwissTopo) show the location of sites that were sampled in Study I. The green circle shows the sampling location that was also sampled in Study II.

Study I: Variability of Estrogenic Activity in Many Independent Rivers[17]

Multiple independent yet similar locations were repeatedly sampled up and downstream from effluent discharges (Fig. 1). Three grab samples were collected in the early months of 2003 (winter) at 19 locations, the interval between the samples was between three and six weeks. Samples were enriched by means of solid-phase extraction (SPE) and the extract was analyzed in the YES, a yeast-based reporter gene assay.[11] The estrogenic activity of the samples was expressed as 17β-estradiol equivalents (EEQs, in ng/l);[18] 17β-estradiol being the standard in the YES. As expected, EEQs in river water were higher downstream from the effluent discharge compared to the paired sample taken upstream. But rather unexpectedly, the average estrogenic activity did not correlate with, for example, the dilution factor of the effluent in the receiving river. Fig. 2a clearly shows that the variability of EEQ values at all sites was quite large (average coefficient of variation, CV = 59%). It was concluded that possibly environmental conditions, but also method issues, contributed to the variability; particularly as the evaluation of the YES is not straightforward.[17,19] With the aim to produce a more robust data set, a second sampling campaign was conducted and 17 locations were sampled again in summer. This time, the three samples were collected with an exact two-week interval (± 3 h). The more uniform sampling regime, along with further method streamlining, was adopted with the aim to reduce variability. The weather during the summer sampling was extremely warm and stable; something which was expected

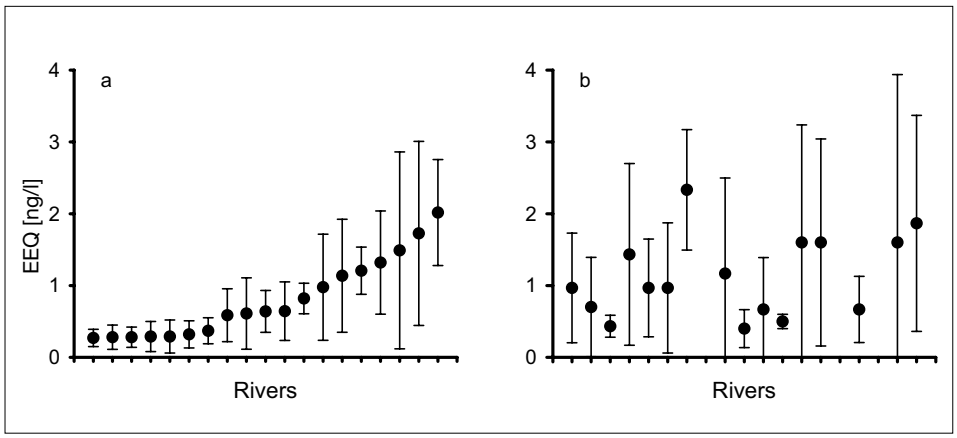

Fig. 2. (a) Average estrogenic activity (EEQ, ng/l; ± standard deviation) of three grab samples collected from 19 rivers in winter downstream from a discharge of treated sewage effluent. Rivers are ordered in ascending average. (b) EEQ data from three samples taken in summer in 17 rivers, ordered as in panel (a). For presentation purposes – in order to keep the 'winter' and 'summer' y-axes to the same range – one summer data point is omitted (7.0 ± 4.4 ng/l).[17]

to support low variability of EEQ values (*i.e.* fairly stable hydrological conditions). Contrary to expectation, the average CV of the second sampling campaign was higher (CV = 79%; P <0.01). As before, average EEQs did not correlate with the proportion of effluent in the river. Neither did average EEQs from winter correlate with average EEQs from summer (Fig. 2ab).[17]

During subsequent statistical analysis of the data it emerged that the variability was unlikely to be a merely random phenomenon. For example, variability correlated between the winter and summer sampling (Fig. 3a). In addition, some 20% of the variability in EEQ values across the sites was 'explained' by river size; smaller rivers being associated with higher variability (Fig. 3b). These observations led to the hypothesis that a relationship may exist between flow rate and EEQ variability in each river. Thus, when EEQs in smaller rivers are more variable than in big rivers, EEQs may become more variable when flow in a river is reduced (the hypothetical Fig. 3c). Obviously this hypothesis requires more support, *i.e.* repeated studies in many independent rivers with good information of flow rate. But, the hypothesis would explain the results that summer EEQ values (rivers at low flow) were more variable than EEQs in winter (higher flow).

Study II: Variability of Estrogenic Activity Around a Single Effluent Source[20]

The aim of Study II was to investigate variability of EEQs in river water and also

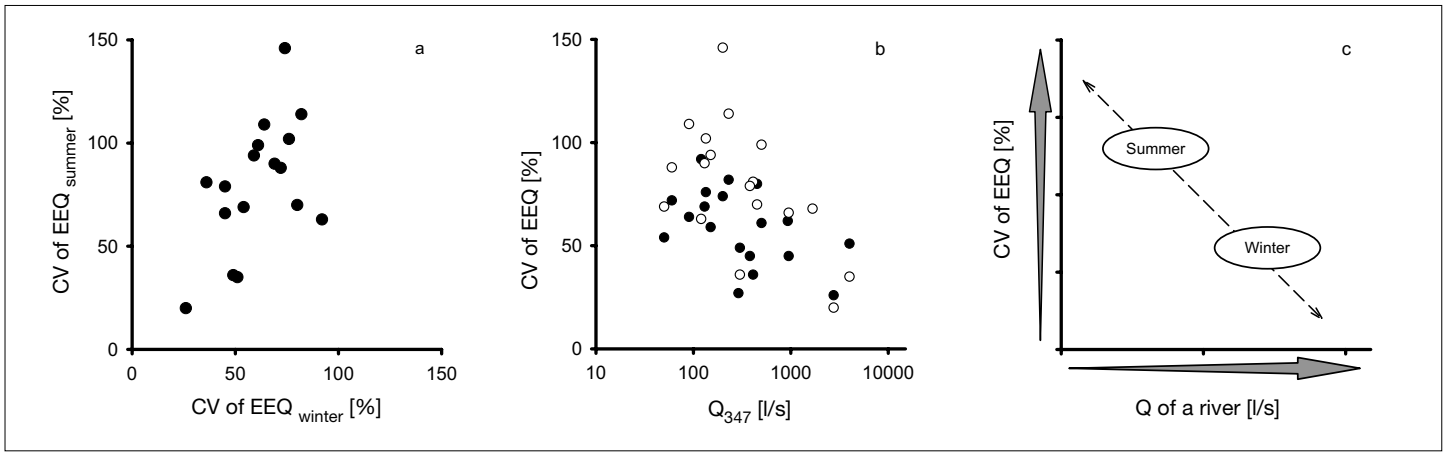

Fig. 3. (a) Association between the coefficient of variation (CV) of the estrogenic activity (EEQ) of three grab samples taken in 'winter' and three samples taken in 'summer'. (b) Association between the CV (winter, filled circles; summer, open circles) of EEQ and the size of the river, given by the Q_{347}, the flow rate exceeded on 347 days in a year. (c) Hypothetical graph that shows how drought (reduction of Q) may be intrinsically linked with an increase in variability of the EEQ values in each river.[17]

effluent in detail at a single site. An important criterion for the site selection was the availability of good data on river and effluent flow rates so that accurate dilution factors could be calculated. The study involved four 12-day sampling blocks that were spaced evenly over the year. Grab samples were taken around 08:00, and as with Study I, enriched with SPE and tested in the YES.

Effluent EEQs varied noticeably over the 48 days, the lowest and highest value differed 11-fold and followed a log-normal distribution (Fig. 4). It cannot be judged if such a log-normal distribution pattern of estrogens in effluent is typical, as no similar studies were found (but see[21]). Nonetheless, it is important to have a well-founded database on the distribution of the estrogenic activity of effluent, especially to calibrate and validate models (*e.g.* the NRP50 project by BMG Engineering: integrative risk assessment for endocrine disruptors in Switzerland).[22]

Effluent EEQs were multiplied with the proportion of effluent in the river downstream from the discharge to calculate expected river EEQs. Measured and expected river EEQs matched rather well;[20] an important observation, as it provided strong support that the methods were appropriate, and that the observed variability in EEQs was not likely to be a method error, neither in Study I nor in Study II. As another link to Study I, it was observed that the average CV of the effluent EEQs was almost double that of the dilution factors (45% *versus* 25%). This indicates that, at least in this river and effluent system, the variability of river EEQs is mainly driven by the variability of the EEQs in the effluent and less so by changing hydrological conditions. It is important to realize that this statement cannot be generalized, as the n in Study II is only one STW/river system; in contrast, Study I involved many independent river systems.

Johnson *et al.*[23] explored the effect of hydraulic retention time (HRT, the time for the effluent to pass through the STW), sludge retention time and temperature on the removal of estrone from effluents of 17 STWs. It was found that a longer HRT is associated with an increased removal of estrone. This is expected from theory[24] and lab experiments with activated sludge solutions show a time-dependent breakdown/transformation of steroidal estrogens.[25] When effluent EEQ data from Study II are plotted against HRT data, an inverse relationship is apparent (Fig. 5); a longer HRT being associated with a lower EEQ. In a general linearized model, HRT emerged as a significant determinant of effluent EEQ ($P < 0.01$).[20] Whereas Johnson *et al.*[23] showed the effect of HRT across sites, Study II showed that HRT clearly determines effluent EEQ at a single site.

Study III: Passive Sampling as a Tool to Assess Estrogenic Activity[26]

In order to overcome the issue of variability, and to produce a more robust measure of the average EEQ values over a certain time window, passive samplers were tested as an alternative sampling approach in Study III. In passive sampling, chemicals partition between the aqueous phase and a sampling phase. Under conditions of constant aqueous concentrations, the concentration of a compound in the sampler increases nearly linearly with time, after which the increase flattens and ultimately the concentrations in the water and sampler reach equilibrium.[27] Particularly the initial – linear – sampling phase can be used to assess time-weighted average aqueous concentrations.[28] Passive samplers for the aquatic environment have been developed and used for almost two decades, but these early samplers (SPMDs, semi permeable membrane devices) were developed to target non-polar contaminants like PCBs and PAHs. In 2004, Petty and co-workers described a

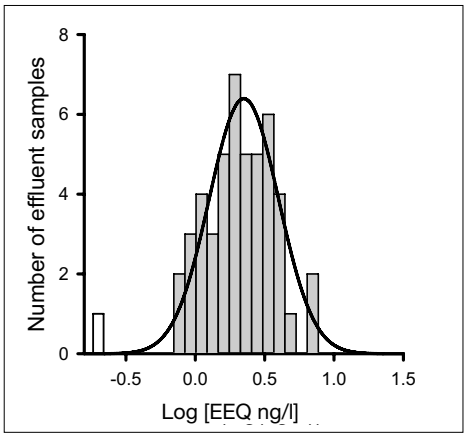

Fig. 4. Histogram of effluent estrogenic activity (EEQ). The normal distribution plot was fitted to 47 data (grey); one EEQ (open bar) was excluded.[20]

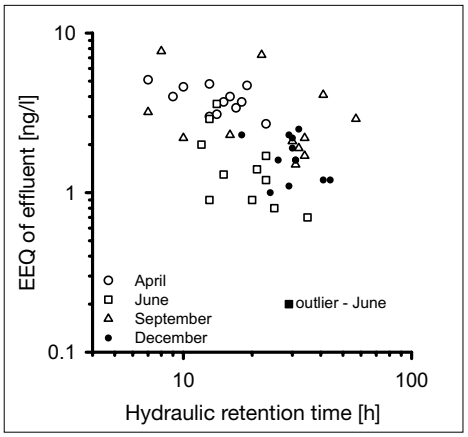

Fig. 5. Relationship between the hydraulic retention time (HRT) and the estrogenic activity (EEQ) of effluent over four 12-day sampling periods. The association between HRT and EEQ was negative in all but one sampling period (small filled circles).[20]

novel sampler type that targets polar organic chemicals – the POCIS (polar organic chemical integrative sampler) – and successfully tested POCIS extracts in the YES.[29] For these reasons, POCIS were selected for a sampling campaign around five STWs.

POCIS were placed upstream and downstream from five discharges and left for 22 days. Then, POCIS extracts were analyzed in the YES and with target analysis of three steroidal estrogens: estrone, 17β-estradiol and 17α-ethinylestradiol. In addition to the passive samples, grab samples were also taken repeatedly at all locations, and caged fish were placed at the downstream sites to assess the accumulation of estrogens in the bile. The study yielded four key observations:[26]

i) POCIS placed downstream from the discharge always showed higher EEQs than the matching POCIS upstream (Fig. 6) – so all discharges were 'identified'. When upstream POCIS already contained significant EEQs, this could be rationalized by the presence of other effluent discharges further upstream.
ii) POCIS EEQs matched grab sample EEQs, showing that the POCIS integrated the fluctuating EEQ concentrations.
iii) EEQs measured in the YES matched calculated EEQs based on chemical analysis of steroidal estrogens multiplied by their relative potency in the YES;[18] confirming the accuracy of the employed methods and identifying estrone as the main compound that is responsible for the EEQ response in the YES.
iv) EEQs in POCIS matched EEQs in the bile of caged fish rather well ($P = 0.07$),[26] given the fact that the n for this particular analysis was only five caging sites.

Taken together, these four observations indicate that POCIS are a biologically relevant and appropriate tool for the assessment of environmental estrogens in river water.

Study IV: Passive Sampling in Many Independent Rivers and Effluents

Based on the very positive results from Study III, a more comprehensive POCIS campaign was initiated with a concept that breaks down to five main points. First, as in Study III, river water was sampled up and downstream from an effluent discharge. This time also the effluent itself was sampled, so it would be possible to match EEQs from effluent with EEQs from river water. To do this, sampling locations had to be selected where good information is available on river flow rates (as in Study II[20]). Second, the chemical analysis of samples was extended beyond the steroidal estrogens to include nonylphenol and bisphenol A, to allow for a more comprehensive comparison between YES and chemical analysis data. Third, the protective housing to deploy the POCIS was redesigned to keep the sampler parallel to the flow and improve the comparability between sampling sites and allow for a direct link with controlled flow experiments. Fourth, water and effluent flow rates and temperature were measured in the field, as the passive sampling process is affected by these environmental parameters. Fifth – linked to points three and four – the effect of flow rate and matrix (river water or effluent) on the passive sampling process was tested experimentally[31] in order to normalize the field data to a standardized flow rate.

The evaluation of the data and some experimental work for Study IV is still ongoing, but a brief overview is provided here. One crucial aspect for this study was the site selection and a main criterion for the location was the proximity of a river flow gauge (Fig. 7a). Furthermore, the rivers had to be sufficiently small, so that they could be easily sampled with three POCIS across their width (Fig. 7b); large rivers are rarely well mixed.[32] Also, the idea was to target rivers with a high effluent load so that sufficient EEQs could be measured downstream from the discharge. This aspect is shown in Fig. 7c as the Q_{347} (the river flow rate exceeded on 347 days in a year) over PE (person equivalent, a measure based on the biological oxygen demand of the influent). Unfortunately, major flood events during Study IV (*e.g.* one river peaked with a 1 in 50 year discharge) led to the loss of many POCIS and the devaluation of the 'river aspect' of Study IV. For this reason a comprehensive data set can only be supplied for the effluents. Twenty-one STWs were between 6000 and 50000 PE, one STW was very large, 180000 PE. All STWs had activated sludge treatment; eight STWs had a sand filter as final treatment.

Triplicate POCIS were placed in the final effluent discharge and left for five weeks, extracted and analyzed for estrogens. All POCIS extracts contained measurable amounts of estrogenic activity, estrone, nonylphenol and bisphenol A; 17β-estradiol and 17α-ethinylestradiol were only rarely detected (as was the case in Study III[26]). Variability of EEQs between the different STWs was large; the lowest and highest EEQ values were 100-fold apart (Fig. 8). It has to be stressed that the data in Fig. 8 have yet to be adjusted for the effect of flow rate on the sampled EEQ amounts. These adjustments are likely to be around two-fold (see next paragraph). The large differences between the effluents are not unexpected as they match results

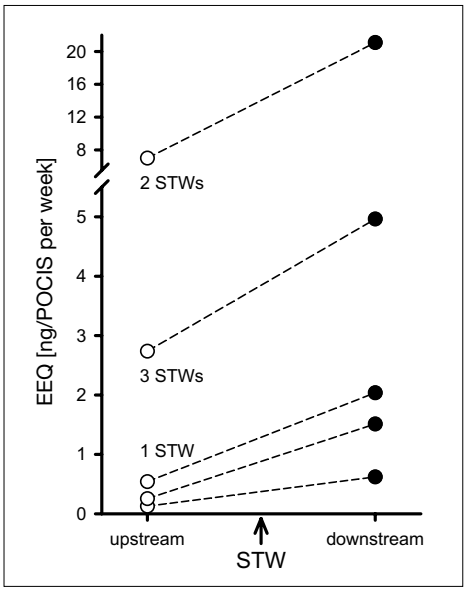

Fig. 6. Estrogenic activity (EEQ) accumulated by (POCIS[30]) placed for 22 days up- (open circles) and downstream from sewage treatment work discharges (STW).[26] Lines connect the up- (open-circles) and downstream sampling sites (filled circles); Numbers in the graph denote the amount of discharges further upstream.

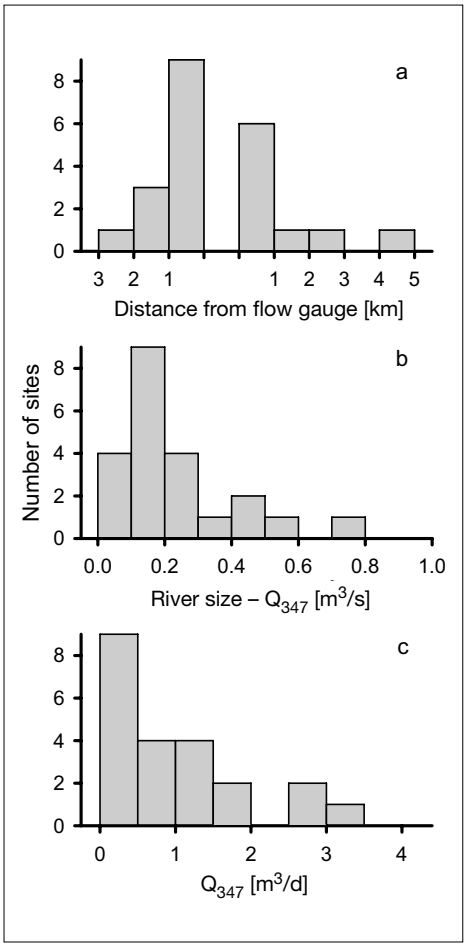

Fig. 7. Characteristics of sampling locations in Study IV. (a) Distance between the flow gauge and the effluent discharge. (b) Q_{347}, the flow exceeded on 347 days in a year, a measure of river size. (c) A measure of effluent dilution: the Q_{347} over the person equivalents (PE) discharging into the river.

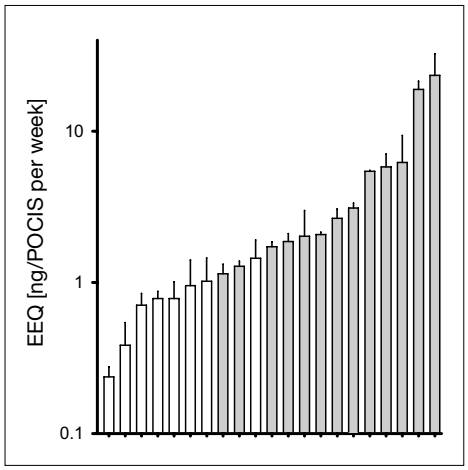

Fig. 8. Estrogenic activity (EEQ) sampled by POCIS[30] from 21 effluents. Open bars show STWs with a sand filter, grey bars show STWs without a sand filter. The standard error is derived from 2–3 POCIS.

shown in Fig. 6. In Study III, the diluted effluent of one STW caused a weekly accumulation of 15 ng EEQ per POCIS (see Fig. 6). So, it is not surprising to find a weekly accumulation rate of 20 ng EEQ per POCIS for the most estrogenic effluent in Study IV. It is also apparent from the data in Fig. 8 that STWs with a sand filter show a particularly good removal rate of estrogenic activity. Interestingly, a recent study did not observe a beneficial effect of a sand filter on the removal of estrogens.[33] This aspect clearly requires further study, for example, by deploying POCIS before and after the filtration step.

In addition to the field work, two channel systems were built to investigate matrix and flow rate effects on passive sampling.[31] The systems run with ambient river water and effluent (*ca.* 20 m^3/h) and mimic riverine flow conditions up to 0.4 m/s. The systems are large enough so the samplers can be deployed in the channels as they are deployed in the field.

Various flow rate trials were performed, particularly with passive samplers based on Empore disks,[34] which also sample estrogenic substances but which have a sampling window of only one to a few days.[31] The matrix did not appear to affect the relationship between flow rate and sampled amounts of chemicals, both for POCIS (unpublished data) and Empore disks.[31] However, the effect of flow rate was quite appreciable, particularly for Empore disks but also for POCIS. When the flow rate increased 15-fold (from 0.025 to 0.37 m/s), sampling rates of Empore disks increased up to five-fold[31] and those of POCIS by about two-fold (unpublished data). These results stress how important it is to understand the effect of flow on the passive sampling process and to combine flow experiments with flow measurements in the field. For future analysis of the data, the results from ongoing channel trials will be used to normalize the field data (Fig. 8) to a standardized flow rate.

Conclusions

As a general observation, it can be stated that the estrogenic activity in Swiss midland rivers is, with a few exceptions, fairly low and comparable to what has been observed in other countries.[13,14] However, in the extensive grab sampling campaigns the large variability of estrogenicity made it difficult to get a confident value of the 'average' load of EEQs in river water. Results from Studies I–III indicate that the variability is not caused by possible method issues, but rather tied to environmental factors and variations in the efficiency of the sewage treatment process. The observed variability complicated the analysis and understanding of the data, then again, these results directed research towards passive sampling. Although research into this area is still very fresh, and passive sampling is certainly not without its uncertainties and drawbacks (*e.g.* influence of environmental factors), the results obtained so far appear quite robust. This is especially the case when passive samplers can be used under fairly controlled conditions such as in an STW, where also the risk of sampler loss is low. But even in rivers, where there is a risk that samplers may be lost, passive samplers are still a tool that can be considered as being superior to repeated grab samples, in particular when one deals with fluctuating concentrations. Once the passive sampler technique has been further developed, and performance reference compounds can be included into the method to control for environmental parameters such as flow rate,[35,36] it will be a significantly superior tool to grab sampling.

Data from Study II, but particularly also Study IV, highlight that it is risky to assume, for modelling approaches, that effluents have a fairly similar EEQ distribution. Although both Study II and IV added to the understanding of EEQ variability in effluents, at the same time these studies stress the fact that there are only very few data sets that cover this topic.

Acknowledgements

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