Review Articles

Effect-Directed Analysis of Key Toxicants in European River Basins A Review

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Abstract

Background. Extensive monitoring programs on chemical contamination are run in many European river basins. With respect to the implementation of the European Union (EU) Water Framework Directive (WFD), these programs are increasingly accompanied by monitoring the ecological status of the river basins. Assuming an impact of chemical contamination on the ecological status, the assignment of effects in aquatic ecosystems to those stressors that cause the effects is a prerequisite for taking political or technical measures to achieve the goals of the WFD. Thus, one focus of present European research is on toxicant identification in European river basins in order to allow for a reduction of toxic pressure on aquatic ecosystems according to the WFD.

Main Features. An overview is presented on studies that were performed to link chemical pollution in European river basins to measurable ecotoxic effects. This includes correlation-based approaches as well as investigations that apply effect-directed analysis (EDA) integrating toxicity testing, fractionation and non-target chemical analysis. Effect-based key toxicants that were identified in European surface waters are compiled and compared to EU priority pollutants. Further needs for research are identified.

Results. Studies on the identification of effect-based key toxicants focused on mutagenicity, aryl hydrocarbon receptor-mediated effects, endocrine disruption, green algae, and invertebrates. The identified pollutants include priority pollutants and other well-known environmental pollutants such as polycyclic aromatic hydrocarbons, polychlorinated dibenzo-*p*-dioxins, furans, and biphenyls, nonylphenol, some pesticides and tributyltin, but also other compounds that were neither considered as environmental pollutants before nor regulated such as substituted phenols, natural or synthetic estrogens and androgens, dinaphthofurans, 2-(2-naphthalenyl)benzothiophene, and N-phenyl-2-naphthylamine.

Discussion. Individual studies at specific sites in a European river basin demonstrated the power of combined biological and chemical analytical approaches and, particularly, of effect-directed analysis. However, the available information on effect-based key toxicants is very limited with respect to the entirety of rivers possibly at risk due to chemical contamination and with respect to toxicological endpoints considered at a specific site. A relatively broad basis of information exists only for estrogenicity and aryl hydrocarbon Ah-receptor-mediated effects.

Conclusions. The development of tools and strategies for an identification of key toxicants on a broader scale are a challenging task for the next years. Since investigations dealing with toxicant identification are too labor and cost-intensive for monitoring purposes, they have to be focused on the key sites in a river basin. These should include hot spots of contamination, particularly if there is evidence that they might pose a risk for downstream areas, but may also involve accumulation zones in the lower reach of a river in order to get an integrated picture on the contamination of the basin.

Recommendations and Perspectives. While EDA is almost exclusively based on measurable effects in *in vitro* and *in vivo* biotests to date, an increasing focus in the future should be on the integration of EDA into Ecological Risk Assessment and on the development of tools to confirm EDA-determined key toxicants as stressors in populations, communities and ecosystems. Considering these requirements and applied in a focused way, toxicant identification may significantly help to implement the Water Framework Directive by providing evidence on the main stressors and possible mitigation measures in order to improve the ecological status of a river ecosystem.

Keywords: Aquatic ecosystems; chemical contamination; ecotoxicity; effect-based key toxicants; effect-directed analysis (EDA); European river basins; sediments; weight-of-evidence; Water Framework Directive (WFD)

Introduction

The European Union (EU) Water Framework Directive (WFD) demands a good ecological status in European river basins by 2015. Together with river morphology and eutrophication, toxic pollution is believed to be an important driving force for the insufficient ecological status of many rivers. Thus, within the last decade, increasing attempts were made to analyze and monitor potentially hazardous compounds in many river basins and numerous individual compounds have been detected and quantified. As an example, polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) and the sulfur analogues of polychlorinated dibenzothiophenes (PCDBTs), polychlorinated biphenyls (PCBs), chlorobenzenes, polybrominated diphenylethers, dichloro-diphenyl-trichloroethane (DDT) and metabolites, hexachlorocyclohexanes, chlorostyrenes, polyaromatic hydrocarbons (PAHs), polycyclic aromatic sulfur hydrocarbons, naphthol, triaz-

ines, phenoxyalkanoic acid pesticides, nitroaromatic compounds, organophosphate pesticides, nonylphenols, nonylphenol ethoxylates, bisphenol A, phthalates, synthetic polycyclic musk fragrances, aromatic amines, chloroalkylphosphates, various pharmaceuticals, organometallic compounds, and heavy metals in the River Elbe basin have been chemically analyzed (Bester et al. 1998, Götz et al. 1998, Stachel et al. 1995, Oxynos et al. 1995, Claus et al. 1998, Heemken et al. 2000, Stachel et al. 2005, Heininger and Claus 1995, Medek et al. 1995, Pietsch et al. 1995, Franke et al. 1995, Gandraß et al. 1995, Börnick et al. 1996, Jantzen and Prange 1995, Wiegel et al. 2004). All of these compounds, as well as many additional ones that have not been included in chemical analyses so far, may impact ecological quality. They are emitted by different sources and demand different measures to reduce the exposure of aquatic communities. Thus, for a successful implementation of the WFD with limited financial resources, it is crucial to identify those contaminants that cause measurable effects as a first step towards an identification and assessment of the driving forces for insufficient ecological quality including chemical and non-chemical stressors.

Triggered by the WFD, chemical monitoring is increasingly accompanied by monitoring the ecological status of the river basins. However, no community-based method is available to date to identify those chemical stressors that affect aquatic organisms *in situ*. The application of bioassays indicating effects on a cellular, organism or population level in laboratory test systems, and the availability of concepts for linking measurable effects of complex environmental samples to distinct toxicants, are required to bridge the gap between chemical contamination and ecological status. Thus, one focus of present European research is on toxicant identification in European river basins in order to allow for a reduction of toxic pressure on aquatic ecosystems according to the WFD (Brack et al. 2005a). As a first step, an overview on attempts to link chemical pollution to ecotoxic effects in European surface waters is required and will be presented in this paper. This includes correlation-based approaches as well as investigations that apply effect-directed analysis (EDA) integrating toxicity testing, fractionation and non-target chemical analysis (Brack 2003). The present review will consider *in vitro* test systems including aryl hydrocarbon Ah-receptor-mediated effects, endocrine disruption, and genotoxicity as well as effects on organisms including bacteria, algae, and invertebrates. The key toxicants that were identified on the basis of these effects are compiled and compared to the list of priority pollutants suggested by the WFD for chemical monitoring.

1 Genotoxicity and Mutagenicity

Since the exposure of bottom dwelling fish to sediment-associated mutagens was suggested to induce hepatic neoplasms, and genotypical and phenotypical alterations, which may lead to tumors, malformation, loss of fertility and immune deficiency (Myers et al. 1991, Vahl et al. 1995), genotoxic and mutagenic compounds are listed as main pollutants by the WFD and should be identified, monitored, and reduced in European surface waters. Several PAHs including the WFD priority pollutants benzo[*a*]pyrene and benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene were frequently identified as important mutagens in sediments (Marvin et al. 1995). EDA in United Kingdom estuary sediment extracts based on Mutatox™ supported the finding of non-polar PAHs as major mutagens (Thomas et al. 2002a). However, attempts to correlate genotoxicity of sediments and suspended matter and organic extracts or aqueous elutriates thereof with PAH concentrations, often fail (Vahl et al. 1997), thus suggesting a contribution of other non-regulated mutagens. In addition to priority pollutants, several non-regulated PAHs could be identified and confirmed by EDA of Neckar basin sediments, including perylene and benzo[*a*]fluoranthene (Brack et al. 2005b). One fraction of outstanding activity did not contain any priority PAHs. This fraction was characterized by three peaks in GC-MS. They were tentatively identified as 11H-indeno[2,1,7-*cde*]pyrene, a methylbenzo[*e*]pyrene and a methyl perylene.

EDA of coastal sediments close to Barcelona based on organic extraction, multi-step fractionation, mutagenicity in the *Salmonella* microsome assay and GC/MS analysis suggested more polar compounds as the cause of mutagenic effects including several polycyclic quinones and nitroquinones, as well as nitro-PAHs including benz[*a*]anthracenequinone, pyrenequinone, nitropyrenequinone, nitroanthraquinone, nitrobenzanthracenedione, 6-nitrochrysene, nitrobenzo[*a*]pyrenes, and nitroindeno[1,2,3-*cd*]pyrene (Fernandez et al. 1992). The relevance of polar fractions for genotoxicity was confirmed for coastal sediments in the River Elbe plume on the basis of the COMET assay in the fish cell line *Epithelioma papulosum cyprini* and embryos of *Danio rerio* (Kammann et al. 2004). Effects were mainly found in the polar fractions and, thus, cannot be explained by polycyclic aromatic hydrocarbons and polychlorinated biphenyls that have been analyzed.

2 Aryl Hydrocarbon-Receptor-Mediated Effects

The binding of xenobiotics to the intracellular Ah receptor triggers numerous adverse effects on many organisms. Cytochrome P450 mono oxygenases are induced that may activate indirect mutagens and trigger cellular proliferation and promotion of mutated cells (Cheung et al. 1996). At least for persistent and planar halogenenated aromatic hydrocarbons such as polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs) and polychlorinated biphenyls (PCBs) Ah-receptor binding induces typical dioxin-like toxicity including reproductive effects, thymic atrophy, body weight loss and acute lethality (Safe 1986). Thus, Ah-receptor binding detected, e.g. by dioxin receptor-chemically activated luciferase gene expression (DR-CALUX) or the induction of ethoxyresorufin-*O*-deethylase (EROD), is a generally agreed marker for the exposure towards potentially hazardous compounds. By clean up, persistent, dioxin-like compounds can be easily separated from non-persistent ones.

In European river basins and adjacent coastal areas, several attempts have been made to identify Ah-receptor mediated toxicants. In Dutch marine harbor sediments, including the Western Scheldt and the Ems estuary, PCDD/Fs and PCBs explained 32 to 90% of the activity of the persistent frac-

tion (Stronkhorst et al. 2002). This clearly suggests these compound groups as key toxicants. Klamer et al. 2005) identified a suite of organic contaminants (PCBs, PAHs, brominated flame retardants, hexachlorobenzene) in DR-CALUX active sediment extracts from the marine end member of the Scheldt estuary. EDA of dioxin-like toxicity revealed dioxins and dibenzofurans as being responsible for ~50% of this type of toxicity in dredged material from the Ems estuary with a clean-up method selecting persistent compounds. Allowing the full range of Ah-receptor mediated toxicants to interact with the receptor by using only a gentle clean up method based on gel permeation chromatography (GPC), only about 7% of the activity could be attributed to PCBs, PCDD/Fs and priority PAHs (Klamer et al. 2004). Thus, the vast majority of Ah-receptors mediated still needs to be identified.

Since the creek Spittelwasser is believed to be one of the major contributors of toxic contamination including dioxin-like compounds, PAHs and many others to the middle and lower Elbe (Götz et al. 1998), extensive EDA has been performed in this creek. EDA of Ah-receptor binding toxicants combined the measurement of ethoxyresorufin-*O*-deethylase (EROD) induction in a rainbow trout liver cell line (RTL-W1) with a multi-step fractionation procedure (Brack et al. 2003) and gas chromatographic analysis with mass specific detection (GC-MS). The fractionation procedure included normal-phase, reversed-phase, electron-donor-acceptor, and size-exclusion chromatography. Major activity was found in a fraction containing non-polar compounds with two aromatic rings (diaromatic fraction) and a PAH fraction co-eluting with chrysene and benz[*a*]anthracene. The activity in the diaromatic fraction was quantitatively explained by PCDD/Fs with 1,2,3,4,7,8-hexachlorodibenzofuran as a major contributor (Brack et al. 2002). The effect of the active PAH fraction was dominated by the non-priority pollutants dinaphtho[2,1-b;2',3'-d]furan, dinaphtho[1,2-b;1',2'-d]furan, 2-(2-naphthalenyl)benzothiophene, 9-methylbenz-[*a*]anthracene, and 1-methylchrysene (Brack and Schirmer 2003). The dinaphthofurans and the naphthalenylbenzothiophenes have been previously reported as by-products of naphthol production (Cermak et al. 1994, Poutsma and Dyer 1982, Guseva et al. 1980). However, they have neither been recognized as environmental contaminants before nor as potent toxicants. In a subsequent study, the dinaphthofurans have been confirmed as Ah-receptor mediated toxicants in the DR-CALUX assay and as estrogen receptor-mediated toxicants in the ER-CALUX (estrogen receptor-mediated, chemically activated luciferase expression) (Vondracek et al. 2004). The dinaphthofurans were also able to release rat liver epithelial WB-F344 cells from contact inhibition at concentrations as low as 100 nM indicating tumor-promoting potential.

Extensive studies on Ah-receptor-mediated effects (CALUX-assay, EROD induction in mammalian H4IIE and fish PHLC-1 cells) of sediment extracts and the analysis of causing compounds have been performed in the Morava River (Czech Republic) as a part of the Danube river basin (Vondracek et al. 2001, Hilscherova et al. 2001, Machala et al. 2001a). The authors found a good correlation between concentrations of analyzed priority PAHs expressed as chemically derived toxicity equivalents and Ah-receptor mediated effects. Sites that were highly contaminated with PAHs also exhibited significant mutagenic activity. Separation into three fractions and subsequent testing for Ah-receptor mediated effects and chemical analysis provided a good agreement between chemically-derived and biologically-derived, toxicity equivalent quantities (TEQs), and indicated that priority PAHs explain most of the effect.

In contrast to the experience in the Morava river basin, Ah-receptor mediated activity of sediment extracts from the Neckar river basin (Germany) (Hollert et al. 2002) and Lake Järnsjön in Sweden (Engwall et al. 1996) could be explained only to a minor portion by priority PAHs. Effect-directed fractionation and analysis of Neckar basin sediments recovered the activity in a PAH fraction that co-elutes with chrysene and benz[*a*]anthracene, although the positive identification of the compounds causing the effect failed (Brack et al. 2005b).

The relevance of PAHs for biochemical responses and effects in fish *in situ* is still not clear. In contrast to *in vitro* effects in exposed chub in the Morava river, PAHs did not significantly contribute to EROD induction (Machala et al. 2001b). In a comparable study in the Anoia river, a tributary to the river Llobregat, EROD activity in carps (*Cyprinus carpio*) and red swamp crayfish (*Procambarus clarkii*) correlated well with the total hydroxylated PAHs determined in the bile ($r = 0.92$; $n = 10$, $P < 0.05$), thus supporting the hypothesis of a relationship between PAH contamination and the biomarker response in fish (Fernandes et al. 2002). However, this relationship was not observed in the case of the Cardener river, where only 9-hydroxyphenanthrene ($r = 0.73$; $n = 9$) and phenylphenol ($r = 0.69$; $n = 9$) showed a statistically significant relationship with EROD activity ($P < 0.05$). This result may also highlight the questionability of such correlation-based approaches. At least *in vitro* phenanthrene, which is metabolized to 9-hydroxyphenanthrene, fails to induce EROD activity in fish cells (Bols et al. 1999). Thus, there is no good reason to assume a cause-effect relationship between this compound and the biomarker response. While *in vitro* responses of PAHs and related metabolizable compounds are unquestionable, further research is obviously required to evaluate and confirm the significance of these compounds on the whole organism level. This demands an extended confirmation of toxicants identified in *in vitro*-based EDA studies in whole organisms and under realistic exposure conditions.

3 Endocrine Disruption

Significant attempts have been made in the last years to detect estrogenic effects in European surface waters and to link them to chemical contaminations. One focus of these investigations was on the Llobregat river basin in Spain, where the presence of estrogenic effects in fish in Spanish waters has been reported for the first time (Solé et al. 2000). In transects upstream and downstream of several sewage treatment plants (STPs) of the two main tributaries of the Llobregat River (Cardener and Anoia), plasma vitellogenin (VTG) levels in male carp (*Cyprinus carpio*) as a biomarker for the exposure to estrogenic compounds correlated with nonylphenol

concentrations in corresponding water samples (r=0.75). This suggested nonylphenol as a possible cause, although the contribution of natural and synthetic estrogens could not be excluded, even if they were found at quantifiable levels in only a few samples. However, the chemical analytical detection limits (2–50 ng/L) were above potential effect levels.

A subsequent, more comprehensive monitoring program, covering a wider range of both compounds and matrices, a longer monitoring period and the measurement of more biological effects at the same sampling sites, should confirm the previously observed estrogenic activity and characterize responsible contaminants (Petrovic et al. 2002). This study revealed the presence of hepatic VTG in up to 60% of the male fish analyzed, high levels of plasma VTG in male carp (higher than in females in some intersexes), sex differences in EROD activity, and a high incidence of intersex fish (close to 40%) in a transect of the Anoia river, downstream to the main STP. Intersex fish were characterized by the coexistence of macroscopically developed ovarian and testicular tissue (gross abnormalities, with fully developed eggs in some of them) and/or testicular atrophy. Pair-wise correlation coefficients between endocrine disrupting compound levels and VTG concentration in male plasma suggested a positive connection (coefficients higher than 0.7) in the case of nonylphenol (r = 0.82, n = 24), total nonylphenolic compounds (r = 0.84, n = 24), estriol (r = 0.78, n = 9) and estrone (r = 0.94, n = 8) in water, and total alkylphenolic compounds (r = 0.83, n = 24) in sediment (Petrovic et al. 2002). These results might indicate a relationship between the contamination with estrogenic compounds and the abnormally high levels of VTG and the intersex fish observed. However, they are not able to prove cause-effect relationships between individual compound levels and the measured effects. Plasma hormonal levels of estradiol (E2) and testosterone (T), as well as levels of the xenobiotic metabolizing capacity in fish liver at sites where increased frequencies of intersex occurred, could not confirm correlations between contamination and response because of the large individual variability (Solé et al. 2003a, Solé et al. 2003b).

In vitro testing of water samples for estrogenicity with a recombinant yeast assay (RYA) confirmed a prevalence of estrogenicity often masked by a hormone-specific, inhibitory activity all through the two rivers and, in basically all cases where estrogens were chemically detected, samples also exhibited clear estrogenic activity (García-Reyero et al. 2001). The *in vitro* system applied was highly sensitive to natural or synthetic estrogens and anti-estrogens present at the ng/L levels, but much less sensitive to nonylphenol with an EC50 value of 80 µg/L and very insensitive to nonylphenol ethoxylates, bisphenol A and phthalates with EC50 values of several mg/L (Céspedes et al. 2004). An approach to link *in vitro* estrogenic responses in the RYA measured in the lower course of the river Llobregat with the contamination with target compounds including octylphenol (OP), nonylphenol (NP), NP mono and di-ethoxylates ($NPEO_1$ and $NPEO_2$), bisphenol A (BPA), phthalates, and synthetic and natural hormones, was done on the basis of summation of estradiol equivalents of the individual compounds (Céspedes et al. 2004, Céspedes et al. 2005). The total load of contaminants increased from upper to downstream reaches, being particularly high in the two sampling points closest to the mouth, which paralleled the data emerging from the RYA analysis (**Fig. 1**). The estrogenicity of the samples expressed as estradiol equivalents and measured by RYA (Bio-E2eq) showed a quasi-linear correlation (r^2 = 0.62) with the estrogenicity calculated from the chemical composition of the samples (Chem-E2eq) (**Fig. 2**) with nonylphenol as a key estrogen. This compound has also been identified as a major contributor to estrogenicity in the rivers Douro and Tejo

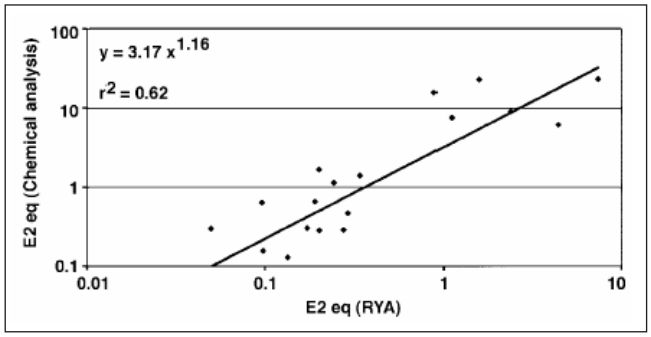

Fig. 2: Correlation between total estrogenicity values (in E2 eq) obtained from RYA (X-axis) and predicted from the chemical composition (Y-axis) in river water samples from the Llobregat River. Note the logarithmic scales and the power regression. (Reproduced from (Céspedes et al. 2005), with permission from Elsevier Science B.V., copyright 2005)

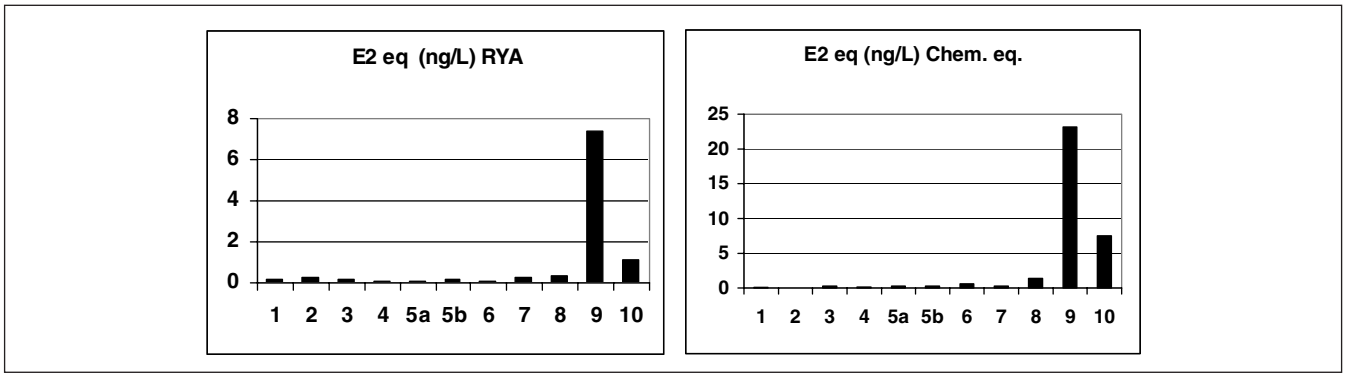

Fig. 1: Total estrogenicity values in estradiol equivalents (E2 eq) measured by RYA (left panel) and calculated from the chemical composition (right panel) in river water samples (1 to 10, see Fig. 2) from the Llobregat river. (Reproduced from (Céspedes et al. 2005), with permission from Elsevier Science B.V., copyright 2005)

close to the cities of Porto and Lisbon (Quirós et al. 2005). However, Chem-E2eq values at the Llobregat overestimated the actual RYA values by a factor of approximately three, on average (Céspedes et al. 2005). Thus, the concentrations of the analyzed compounds were obviously high enough to induce estrogenicity as found in the river water. However, an actual quantitative agreement was not found. The results did not allow an exclusion of non-measured compounds as relevant for the total effect. An actual quantification of the contribution of individual compounds to the measured effect of the water samples was not possible.

The challenge of considering non-detected compounds in quantitative assessment of estrogenic compounds in complex environmental samples has also been demonstrated in a study in the catchment area of the River Neckar (Hollert et al. 2005). Those estrogenic compounds that were analyzed in concentrations above the detection limits, including nonyl and octylphenol, phthalates, PCBs, bisphenol A, and DDT, were able to explain 9 to 14% of the total Bio-E2eq with nonylphenol as a major contributor. For natural and synthetic hormones that could not be detected, such as 17β-estradiol and ethinylestradiol, concentrations of 0 ng/L were assumed. If concentrations of these compounds close to the detection limits were taken as a basis of estimation, 95% of the Chem-E2eq were contributed by ethinylestradiol and 17β-estradiol with a total Chem-E2eq exceeding the Bio-E2eq by 40 to 100%. Advanced analytical methods for natural and synthetic hormones with lower detection limits are one way to reduce this problem (Aerni et al. 2004).

Another problem related to the estradiol-equivalent approach was reported for the assessment of estrogenicity in Swiss river waters with the yeast estrogen screen (YES) (Vermeirssen et al. 2005). Estradiol-equivalents obviously did not dilute parallel with the samples. However, the dilutability and additivity of these equivalents are the basis for the whole concept. In contradiction to the mentioned study, Silva et al. (2002) demonstrated the validity of the concept of concentration addition for a mixture of eight weak estrogens in the YES assay.

For several sites from the river basins of the Neckar, Rhine (Germany) and Thames (Great Britain), an EDA approach based on passive sampling with SPMDs, highly resolved reversed-phase HPLC fractionation and a YES could show that estrogenicity patterns may be rather site-specific with most active fractions within a log K_{OW} window of 3.16 to 7.23 (Rastall et al. 2006). In addition to nonylphenol isomers, several candidate compounds for estrogenicity could be identified in active fractions including benzophenone, phthalates, dehydroabietic acid, sitosterol, 3-(4-methylbenzylidine)camphor, and 6-acetyl-1,1,2,4,4,7-hexamethyltetralin. A confirmation of those compounds as the cause of measured effects is still to be performed.

Extensive EDA of estrogenicity in United Kingdom estuaries has been performed combining solid phase extraction (SPE) of water samples and RP-HPLC fractionation with YES (Thomas et al. 2001, Thomas et al. 2002b). The natural steroid hormone 17β-estradiol derived from domestic sewage treatment works explained about 84 to 90% of the *in vitro* activity of concentrates of estuarine surface waters. Minor contributions could be assigned to nonylphenol and phthalates. This is in good agreement with combined biological and chemical assessment of estrogenicity in Swiss wastewater treatment plant effluents and receiving waters (Aerni et al. 2004), as well as with an EDA study of estrogenic compounds in deconjugated bile of male breams from different rivers in The Netherlands (Houtman et al. 2004). Both studies identified the natural hormones 17β-estradiol, estrone, and estriol as major contributors to estrogenicity. At one site (river Dommel), the synthetic contraceptive pill component ethynylestradiol was found in effective concentrations as well. In parallel to estrogenicity, several naturally-produced steroids, including dehydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α, 11β-diol-17-one, androsterone, and epi-androsterone, could be identified in United Kingdom estuaries as being responsible for 99% of *in vitro* androgenicity in a yeast androgen screen (YAS) (Thomas et al. 2002b). This was the first time that natural steroidal androgens have been reported as potential contaminants in the aquatic environment. In the Elbe River, at several sites, tributyltin could be identified to be responsible for androgenic effects in snails (Schulte-Oehlmann et al. 2001).

4 Toxicity to Luminescent Bacteria

Since bioluminescence of *Vibrio fischeri* is a standardized, easy to handle and frequently applied tool for testing individual compounds and environmental samples for cytotoxicity, it was also applied in EDA studies. In the river Elbe, chemical analysis of sediment extracts from four sites was combined with primary fractionation on aluminum oxide and toxicity testing. The authors found a strong correlation between extract toxicity and elemental sulfur content. After sulfur elimination, the toxicity of extracts and fractions was dramatically reduced. The remaining toxicity was rather evenly distributed over the fractions (Heininger et al. 1998). Similar results have been achieved by (Reineke et al. 2002) with River Elbe surface water sampled in Hamburg. This study combined toxicity testing with *Vibrio fischeri* with solid-phase extraction, reversed-phase fractionation and GC-MS analysis. The authors found a relatively even distribution of toxicity over the fractions with highest activities in moderately hydrophobic fractions characterized by caffeine, desethylatrazine and linuron. Sub-fractionation of one of the active primary fractions and subsequent testing provided a similar picture. Thus, no individual fractions could be identified as a major problem and no toxicants could be confirmed as the causes of measured effects. Both studies indicate a low discriminative power of the applied test system and, thus, the limited value as a test system in EDA studies. *Vibrio fischeri* detects, particularly, non-specific narcotic effects that are exhibited by all compounds, increasing with higher hydrophobicity. In surface waters, a tremendous variety of natural and anthropogenic compounds at low concentrations is generally present. All of them contribute to this baseline toxicity while specific effects, e.g. of pesticides, are not detected by this test system (Brack 2003). One of a few compounds that are known for specific effects in the

acute bioluminescence inhibition test is elemental sulfur that is an intermediate in the natural sulfur cycle and not in the focus of EDA. In North Sea water samples, where anthropogenic compounds are present in relatively low concentrations, the biogenic compound 4-bromophenol explained about 25% of *Vibrio fischeri* toxicity of concentrated extracts (Biselli et al. 2005).

5 Toxicity to Green Algae and Invertebrates

For the detection and identification of phytotoxicity in contaminated samples, cell multiplication of green algae is an appropriate and frequently used endpoint. It was successfully applied to sediment extracts from the creek Spittelwasser as one of the major contributors of toxic contamination of the middle and lower Elbe (Brack et al. 1999, Grote et al. 2005a). The compounds N-phenyl-2-naphthylamine, prometryn, tributyltin, and PAHs were identified and confirmed by EDA as the dominating toxicants to *Scenedesmus vacuolatus*. Despite its specifically high toxicity to green algae, N-phenyl-2-naphthylamine has not previously been reported as a relevant environmental contaminant. The strong inhibition of algal growth by PAHs, that are frequent contaminants of aquatic sediments in industrialized regions, could be identified as phototoxicity due to minor emissions of UV radiation, even under standard algal growth light (Grote et al. 2005b). At the same site, a high toxicity of sediment extracts to *Daphnia magna*, as a frequently used representative for aquatic invertebrates, could also be detected and assigned to the insecticide methyl parathion and tributyltin (Brack et al. 1999).

Extensive identification of toxicants towards the marine copepod *Tisbe battagliai* applying RP-HPLC fractionation and GC-MS analysis has been performed in United Kingdom estuaries. Candidate toxicants that were identified in active fractions include tri-, tetra-, and pentachlorophenol, 4-chloro-3,5-dimethylphenol, nonylphenol, 4-chloro-3,5-xylenol, dieldrin, atrazine, and carbophenothion methylsulfoxide. However, these compounds are not confirmed yet as the cause of measured effects and, in several toxic fractions, GC-MS was not able to detect and identify any compound (Thomas et al. 1999).

6 Identified Key Toxicants

Contaminants that have been identified as key toxicants at different sites in European river basins in studies focusing on cause-effect relationships between chemical contamination and biological effects are summarized in **Table 1** and compared to the priority pollutant list provided by the European Commission.

7 Summary and Conclusions

Chemical analysis of pre-selected sets of toxicants (e.g. priority pollutants) is often not able to explain ecotoxic effects of complex environmental samples. Risk assessment based on concentrations, e.g. of priority pollutants in sediments or water, obviously does not reflect the risk of the actual mixture of contaminants, but only the risk of those pre-selected toxicants. All other compounds are ignored. The approach does not provide any information on the quality of the risk assessment in terms of how much of the actual risk was really considered. Thus, combined biological and chemical-analytical approaches provide an important progress towards an identification of those toxicants that are relevant for site-specific risks and towards an estimation of the portion of an effect that can be explained by the analyzed chemicals. Approaches based on the correlation between concentrations of individual compounds and measurable effects in typically complex environmental mixtures generally do not provide reliable cause-effect relationships. In contrast, estimations of the contribution of chemically analyzed compounds to the total, measured effect of the mixture on the basis of compound-specific effect data and concentration, addition as a concept for mixture toxicity may be quite successful in estimating the contribution of selected compounds to the total effect. This holds particularly for responses triggered by the binding to a specific receptor such as the estrogen or the Ah-receptor with a limited number of compounds often explaining high portions of the effect. However, even for those effects a quantitative agreement between chemically derived effect estimations and measured effects as a crucial basis for reliable conclusions is not always achievable. The approach does not provide a possibility to identify unknown causes of effects. The most promising approach to solve this problem is the sequential combination of toxic syndrome-related bioassays, fractionation procedures and chemical analysis referred to as EDA. The potential of this approach was shown, e.g. in several studies in the industrial area of Bitterfeld in the Elbe basin and in UK estuaries. Prerequisites for a successful application of the approach are (1) significant concentrations of specifically acting toxicants rather than an even distribution of potential toxicity over high numbers of compounds in very low concentrations, as it may be observed in samples taken far from pollution sources and (2) the use of a toxicological endpoint that allows the detection of specific effects, rather than only baseline toxicity such as bioluminescence of *Vibrio fischeri*.

Effect-based key toxicants that have been identified to date include priority pollutants such as polycyclic aromatic hydrocarbons, tributyltin, and nonylphenols, as well as compounds that have not been considered as environmental pollutants so far such as n-phenyl-2-naphthylamine, dinaphthofurans and naphthalenylbenzothiophene. A reliable estimation of the contribution of priority and non-regulated or unexpected pollutants to environmental risks seems to be impossible on the basis of the available data set. However, there are clear indications that the impact of non-regulated toxicants is significant and may even dominate effects and risks. Thus, the development of powerful, but also routinely applicable tools for the identification of effect and risk-based key toxicants, as well as the identification of basin and site-specific key toxicants, are important challenges for the future. To date, attempts to identify effect-based key toxicants in European river basins have been rare and scattered. Most studies focused on few sites and one or few ecotoxicological endpoints. Strategies for an identification of key toxicants on a broader scale still need to be developed. This is also of great importance for ecological risk assessment on the basis of Weight-of Evidence approaches that increasingly tend to include causation, in addition to correlation-based lines of evidence (Chapman and Hollert 2006). An integration of

Table 1: Key toxicants identified in European river basins

Compound	Priority substance [a]	Confirmed [b]	Site/basin	Reference
Mutagenicity/genotoxicity				
benzo[*a*]pyrene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, benzo[ghi]perylene	Yes	Yes	Many sites worldwide	e.g. (Brack et al. 2005b)
perylene, benz[*a*]fluoranthene	No	Yes	Neckar basin (Germany)	Brack et al. 2005b
11H-indeno[2,1,7-*cde*]pyrene, methyl benz[*e*]anthracenes and perylenes	No	No	Neckar basin (Germany)	Brack et al. 2005b
polar polycyclic compounds including benz[*a*]anthracenquinone, pyrenequinone, nitropyrenequinone, nitroanthraquinone, nitrobenzanthracenedione, 6-nitrochrysene, nitrobenzo[*a*]-pyrenes, nitroindeno[1,2,3-*cd*]pyrene	No	No	Mediterranean Sea, coastal zone at Barcelona (Spain)	(Fernandez et al. 1992)
Ah-receptor-mediated effects				
PCDD/Fs, PCBs	No	Yes	Western Scheldt (The Netherlands), Spittelwasser (Elbe basin, Germany)	(Stronkhorst et al. 2002, Klamer et al. 2005, Brack et al. 2002)
benzo[*a*]pyrene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene	Yes	Yes	Morava river (Danube basin, Czech Republic)	(Vondracek et al. 2004, Hilscherova et al. 2001, Machala et al. 2001c)
dinaphthofurans, 2-(2-naphthalenyl)benzothiophene, 9-methylbenz[*a*]anthracene, 1-methylchrysene	No	Yes	Spittelwasser (Elbe basin, Germany)	(Brack and Schirmer 2003)
Estrogenicity				
nonylphenol	Yes	Yes	Llobregat (Spain), river Neckar	(Céspedes et al. 2005, Hollert et al. 2005)
benzophenone, phthalates, dehydroabietic acid, sitosterol, 3-(4-methylbenzylidine)camphor, 6-acetyl-1,1,2,4,4,7-hexa-methyltetralin	No	No	Rivers Neckar, Rhine (Germany), Thames (United Kingdom)	(Rastall et al. 2006)
tributyltin	Yes	Yes	Elbe (Germany)	(Brack et al. 1999, Schulte-Oehlmann et al. 2001)
17β-estradiol, estrone, estriol	No	Yes	United Kingdom estuaries, different rivers in the Netherlands, Swiss wastewater treatment plant effluents	(Thomas et al. 2001, Houtman et al. 2004, Aerni et al. 2004)
Androgenicity				
dehydrotestosterone, androstenedione, androstanedione, 5β-androstane-3α,11β-diol-17-one, androsterone, epi-androsterone	No	Yes	United Kingdom estuaries	(Thomas et al. 2002b)
Green algae				
N-phenyl-2-naphthylamine, prometryn	No	Yes	Spittelwasser (Elbe basin, Germany)	(Brack et al. 1999)
priority PAHs, tributyltin	Yes	Yes	Spittelwasser (Elbe basin, Germany)	(Brack et al. 1999)
Invertebrates				
methyl parathion	No	Yes	Spittelwasser (Elbe basin, Germany)	(Brack et al. 1999)
pentachlorophenol, atrazine	Yes	No	United Kingdom estuaries	(Thomas et al. 1999)
tri-, tetra-chlorphenol, 4-chloro-3,5-dimethylphenol, nonylphenol, 4-chloro-3,5-xylenol, dieldrin, carbophenothion methylsulfoxide	No	No	United Kingdom estuaries	(Thomas et al. 1999)

[a] according to EU-WFD
[b] confirmed as a cause of the measured effect

EDA-like approaches with *in situ* community assessment combines the relative ease of interpretation and the analytical power of EDA-like approaches with the high ecological relevance of field studies (**Fig. 3**).

Since investigations dealing with toxicant identification are too labor and cost-intensive for monitoring purposes, they have to be focused on the key sites in a river basin. These should include hot spots of contamination, particularly if there is evidence that they might pose a risk for downstream areas, but there may also be accumulation zones in the lower reach of a river in order to get an integrated picture on the contamination of the basin. While EDA is based on measurable effects in *in vitro* and *in vivo* biotests to date, an increasing focus in the future should be on the integration of EDA into ecological risk assessment and on the development of tools to confirm EDA-determined key toxicants as stressors in populations, communities and ecosystems. This would increase the field relevance without loosing analytical power. Considering these

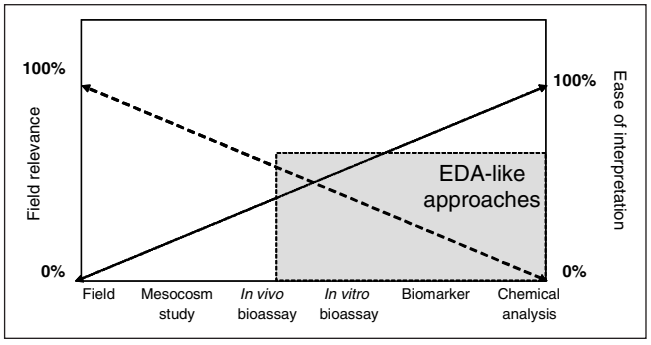

Fig. 3: Cross scheme showing an increasing ease of interpretation from field studies on community health via biological analysis in vivo and in vitro towards chemical analysis (dotted line) paralleled by a loss of field relevance (broken line). The strength of EDA-like approaches is more the analytical power and ease of interpretation than field relevance

requirements and applied in a focused way, toxicant identification may significantly help to implement the Water Framework Directive by providing evidence on the main stressors and possible mitigation measures in order to improve the ecological status of a river ecosystem.

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