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Final report

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Validation of the NSAID in vitro assay for biomonitoring of NSAID activities in surface waters

Final report

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Abstract: Validation of the NSAID *in vitro* assay for biomonitoring of NSAID activities in surface waters

The NSAID *in vitro* assay developed as a tool for environmental monitoring of pharmaceutical effects was validated for its suitability to detect COX inhibitor activity in surface water. A standard operation procedure based on solid phase extraction of aqueous samples was established by modifying a sampling protocol for the detection of estrogens with the aim to stabilize NSAIDs in the samples. In addition, the sensitivity of the NSAID *in vitro* assay was improved by minimizing matrix effects and improving the stability of the substrate arachidonic acid. The validation characteristics linearity, measurement range, accuracy, precision and limit of detection were evaluated. The test procedure was used for measuring 39 surface water samples taken at EU Watch List sampling sites in 14 EU member states and 4 Swiss cantons between autumn 2017 to spring 2018. Diclofenac equivalents measured by the NSAID in vitro assay were compared with data obtained by chemical analysis (LC-MS/MS) of diclofenac and other COX-inhibitors.

Kurzbeschreibung: Validierung des NSAID *in vitro* Assays zum Nachweis von NSAID Aktivitäten in Oberflächenwasser

Der als Werkzeug für die Umweltüberwachung von Arzneimitteln entwickelte NSAID *in-vitro* Assay wurde hinsichtlich seiner Eignung zum Nachweis von COX-Inhibitor-Aktivitäten in Oberflächengewässern validiert. Es wurde ein Standardverfahren basierend auf der Festphasenextraktion von wässrigen Proben etabliert. Hierzu wurde ein Probenahmeprotokoll, das für Östrogene eingesetzt wird, so modifiziert, dass eine höhere Stabilität von NSAIDs erreicht wurde. Die Sensitivität des NSAID *in-vitro* Assays wurde verbessert, indem Matrixeffekte minimiert und die Stabilität des Substrats Arachidonsäure erhöht wurde. Die Validierungsmerkmale Linearität, Messbereich, Genauigkeit, Präzision, und Nachweisgrenze wurden bestimmt. Das Testverfahren wurde für die Messung von 39 Oberflächenwasserproben, die zwischen Herbst 2017 und Frühjahr 2018 an EU Watch List Probenahmestellen in 14 EU-Mitgliedstaaten und 4 Schweizer Kantonen entnommen wurden, eingesetzt. Die mit dem NSAID *in vitro* Assay gemessenen Diclofenac-Äquivalente der Oberflächenwasserproben wurden mit den Ergebnissen der chemischen Analytik (LC-MS/MS) von Diclofenac und anderen COX-Inhibitoren verglichen.

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COX	Cyclooxygenase
dicEQ	Diclofenac equivalent
DTT	1,4 Dithiothreitol
EC50	Half maximal effect concentration
EDTA	Ethylenediaminetetraacetic acid
EDA	Effect-directed analysis
FCS	Fetal calf serum
FB	Field blank
GFP	Green fluorescent protein
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
LC	Liquid chromatography
LoD	Limit of detection
LogD	Logarithm of the distribution coefficient
LoQ	Limit of quantitation
MOA	Mode of action
MS	Mass spectroscopy
MTBE	Methyl tert-butyl ether
NSAID	Non-steroidal anti-inflammatory drugs
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity
Ro GFP	Redox active GFP
SOP	Standard operation procedure
SPE	Solid-phase extraction
SPE c.	SPE concentrated
v.c.	Vacuum concentrated
WFD	Water Framework Directive

Summary

Background

The aquatic environment is exposed to the input of contaminants from the anthropogenic origin. Due to the use, numerous pharmaceuticals are released into the wastewater. Many of those pharmaceuticals are far from being effectively degraded by conventional wastewater treatment. In consequence, increasing amounts of these compounds reach streams and lakes which often serve as drinking water reservoirs in industrialized countries. In this context, environmental contamination by pharmaceuticals has become an issue of great concern to environmental policy, particularly in the European Union. Within the EU Water Framework Directive implementation process, diclofenac was placed on a watch list for which EU-wide monitoring data were gathered. Diclofenac and other painkiller belonging to the group of nonsteroidal anti-inflammatory drugs (NSAIDs) exert their analgesic action through inhibition of prostaglandin synthesis by inhibition of cyclooxygenases. Due to the phylogenetic conservation of central signal transduction pathways among vertebrates, NSAIDs affect vertebrate species in particular fish even at low concentrations. Analytical monitoring surveys routinely measure concentrations of only single compounds. To decide, whether NSAIDs pose risks to organisms in aquatic environments, one has to be aware of mixture effects of all compounds, metabolites, and transformation products with the same mode of action (MOA). Complex mixtures of compounds with the same MOA could lead to physiological responses that deviate quantitatively from the additive physiological responses of the individual compounds. Therefore, a fast responding MOA-directed fluorescence-based NSAID *in vitro* assay was developed using a biosensor cell line allowing the specific and sensitive quantification of cyclooxygenase inhibition and the estimation of a resulting environmental impact of complex mixtures.

Objective

The aim was to investigate the applicability of the NSAID *in vitro* assay for monitoring cyclooxygenase inhibition activity of environmental probes. A monitoring project was initiated by the Swiss Centre for Applied Ecotoxicology (Eawag) and the German Environment Agency (UBA) to investigate the applicability of effect-based methods for monitoring steroidal estrogens and NSAIDs. Samples taken at the sampling sites of the European water framework watch list in 14 EU member states and 4 Swiss cantons were shipped directly to BioDetection Systems (NL), where sample preparation and distribution was performed. The aim was to detect the presence and activity of both estrogenic compounds and NSAIDs by chemical analysis (LC-MS/MS) and effect-based methods in surface waters. More specifically, the objectives of this study were

- to prove the established estrogen sampling protocol for influencing NSAID stability;
- to asses possible matrix effects;
- to adapt and optimize the NSAID *in vitro* assay for a cost-efficient screening with maximal sensitivity and robustness;
- to validate the optimized NSAID *in vitro* assay;
- to measure NSAID activity of Watch list samples by the optimized NSAID *in vitro* assay and to compare effect-based and chemical analytical methods;

Results

Modification of the sampling protocol

The standard operation protocol developed for estrogens could not be used for the sampling of NSAIDs due to NSAID instability in aluminum bottles at pH 3 measured during preliminary experiments. Therefore, an alternative sampling method was developed. Here we decided to use aluminum bottles for sampling without acidification. A standard operating procedure based on solid-phase extraction of 700 mL of an aqueous sample was developed. The mid- and long-term stabilities of NSAIDs were evaluated. With the exception of ketoprofen, a noticeable loss was observed after 24 weeks of storage for the tested NSAIDs diclofenac, ibuprofen, indomethacin and naproxen. It was therefore decided that the water samples should be stored for a maximum period of 4 weeks at 4°C after sampling.

Adaptation and optimizing of the NSAID *in vitro* assay

The NSAID *in vitro* assay was adapted to fulfill the required maximum acceptable method detection limit for the watch list monitoring (≤ 50% of the envisaged environmental quality standard of 0.05 µg/L diclofenac) and optimized for maximal sensitivity and robustness. Several arachidonic acid preparations were examined with regard to their suitability to function as a substrate for the cyclooxygenase expressed in the NSAID sensor cell. The arachidonic acid sample A3611 (Sigma-Aldrich, > 98 % purity) showed a significantly higher cyclooxygenase substrate activity compared to a similar product with a purity > 95% (Sigma-Aldrich A10931). This product was therefore used in all other experiments. The autoxidation of arachidonic acid was minimized by dissolving pure arachidonic acid in water-free DMSO saturated with nitrogen and by diluting this stock solution in HEPES buffer immediately before starting the assay. An assay protocol for the optimized NSAID *in vitro* assay was developed.

Validation of the optimized NSAID *in vitro* assay

The validation was directed to an analytical procedure to quantitatively analyze cyclooxygenase inhibition activity of NSAIDs in complex mixtures of surface water samples in the concentration range of the envisaged diclofenac environmental quality standard. The validation characteristics linearity, range, accuracy, precision, and limit of detection were evaluated.

Linearity:

The concentration-response curve of the cyclooxygenase inhibition biosensor assay shows a common inhibitor versus response shape. The curve could be fitted using a variable slope 4-parameter sigmoidal fitting. The concentration of the cyclooxygenase inhibitors that gives a response half between bottom and top of the sigmoidal curve, defined as half-maximal diclofenac equivalent effect concentration was calculated to be 2.4 µg/L.

Range:

The interval between the upper and lower concentration of the analyte diclofenac for which the NSAID *in vitro* assay has a suitable level of linearity was estimated to be 1.2 – 7 µg/L. A concentration process of the environmental samples by a factor of 140 resulted in a sensitivity suitable for reaching the required limit of detection.

Accuracy:

The closeness of agreement between the value accepted as a true value and the value found was determined by spiking diclofenac into samples. The recovery of spiked diclofenac for three concentrations varied between 95 and 105 %. The accuracy was proved to be sufficient with a relative standard deviation of 2 – 8 %.

Precision:

The repeatability of 8 replicates containing 3.6 µg/L diclofenac was demonstrated showing a relative standard deviation of 8%.

Limit of detection:
For non-concentrated samples, the limit of detection was calculated from the triple of the noise signal standard deviation to be 0.9 µg/L. The solid-phase extraction was performed in such a way that after the resolution of the dry residue a concentration by a factor of 140 was achieved. This resulted in a limit of detection for the entire analytical procedure of 6 ng/L. The NSAID in vitro assay shows a similar sensitivity as to chemical analysis with a limit of quantification of 12 ng/L.

NSAID activity in the Watch List samples
Only 39 out of 71 surface water samples could be measured due to inadequate storage during transport by a third party and the resulting loss of substance. Samples and 5 field blanks were analyzed for diclofenac along with other cyclooxygenase inhibitors by LC-MS/MS and screened for cyclooxygenase inhibition by the optimized NSAID *in vitro* assay. For most of the samples, the diclofenac equivalent was slightly higher than the diclofenac concentration measured by LC-MS/MS. The cyclooxygenase inhibitors fenoprofen, ibuprofen, indomethacin, ketoprofen, naproxen, paracetamol, and salicylic acid could not be detected in significant quantities by LC-MS/MS. Therefore, we conclude that the NSAID activity of the environmental samples tested was mainly caused by diclofenac. 14 out of 39 (36%; LC-MS/MS) and 16 out of 39 (41%; NSAID in vitro bioassay) environmental probes exceeded the actually envisaged environmental quality standard for diclofenac of 0.05 µg/L[1]

Alternative sample concentration
For the majority of tested NSAID compounds, a good extraction efficiency between 80% and 100% could be achieved by the developed solid-phase extraction (SPE). However, surface waters can contain very different matrix components, which can affect SPE. In such cases, isotopically labeled internal standards compensate for losses and still ensure a reliable quantification by LC-MS/MS. In contrast to the chemical analysis procedure, reduced extraction efficiency during SPE leads to a reduced absolute dicEQ amount measured by the NSAID *in vitro* assay. In order to avoid a loss of NSAIDs due to reduced extraction efficiency and to ensure the measurement of the complete complex mixture, an alternative concentration method was developed. A vacuum concentration process was successfully adapted as an alternative. Water samples upstream and downstream of a sewage treatment plant effluent as well as the respective effluent sample were analyzed by LC-MS/MS and the NSAID *in vitro* assay. All analysis data correlated well. Therefore, for the measurement of dicEQs, we recommend a concentration procedure of surface water samples by vacuum concentration.

Conclusions
The optimized NSAID *in vitro* assay with a detection limit of 6 ng/L diclofenac fulfills the required maximum acceptable method detection limit for the EU Watch List monitoring. The accuracy and precision are of sufficient quality. High-quality monitoring data were produced and the applicability was shown to complement targeted monitoring of NSAIDs in the aquatic environment under the EU Water Framework Directive. The applied effect-based method for the quantification of NSAID activities shows a comparable sensitivity to chemical analytics pointing out its good screening potential. Chemical analytical and effect-based methods for NSAID quantification can be combined to get benefits of both:

- to receive an indication on substances and their contribution to the measured effects
- to study variable exposure of complex mixtures

[1] EQS Datasheet, Environmental Quality Standard Diclofenac
German Environment Agency (UBA), 2017

- to exclude false negative measurements
- to monitor NSAIDs in the aquatic environment

Zusammenfassung

Hintergrund

Die aquatische Umwelt ist dem Eintrag von Schadstoffen aus anthropogenem Ursprung ausgesetzt. Nach der Einnahme wird ein Großteil der Arzneimittel vom Menschen in das Abwasser ausgeschieden. Viele dieser Medikamente werden durch die konventionelle Abwasserbehandlung nicht effektiv abgebaut. Dadurch erreichen immer mehr dieser Verbindungen Flüsse und Seen, die in den Industrieländern auch als Trinkwasserspeicher genutzt werden. Für die Umweltpolitik, insbesondere in der Europäischen Union ist die Kontamination der Umwelt mit Arzneimittel von großer Bedeutung. Im Rahmen der Umsetzung der EU-Wasserrahmenrichtlinie wurde Diclofenac auf die „Watch List" gesetzt, für die EU-weite Überwachungsdaten erhoben wurden. Diclofenac und andere Schmerzmittel aus der Gruppe der nicht-steroidalen entzündungshemmenden Medikamente (NSAIDs) üben ihre analgetische Wirkung durch Hemmung der Prostaglandinsynthese über die Hemmung von Cyclooxygenasen aus. Aufgrund der phylogenetischen Konservierung zentraler Signaltransduktionswege bei Wirbeltieren wirken NSAIDs auf Wirbeltierarten insbesondere auf Fische auch in niedrigen Konzentrationen. Für eine vollständige Risikoeinschätzung von Gewässern bezüglich der Belastung durch NSAIDs muss man Verbindungen, Metaboliten und Transformationsprodukte mit dem gleichen Wirkprinzip (MOA) sowie Mischungseffekte zusammen berücksichtigen. Komplexe Mischungen von Verbindungen mit dem gleichen MOA können zu physiologischen Reaktionen führen, die quantitativ von den additiven physiologischen Reaktionen der einzelnen Verbindungen abweichen. Im Rahmen der Überwachung von Gewässern werden routinemäßig allerdings nur die Konzentrationen von Einzelverbindungen analysiert. Daher wurde ein MOA-basierter NSAID *in vitro* Test entwickelt, der über eine Biosensor-Zelllinie fluoreszenzbasiert eine spezifische und sensitive Hemmung der Cyclooxigenase und damit die Einschätzung einer Belastung von komplexen Mischungen ermöglicht.

Zielsetzung

Ziel dieser Arbeit war, die Anwendbarkeit des NSAID *in vitro* Assays zur Überwachung von Watch List Umweltproben zu untersuchen. Das Schweizer Oekotoxzentrum (Eawag) und das Umweltbundesamt (UBA) initiierten ein Monitoring, um die Anwendbarkeit von effektbasierten Methoden zur Überwachung steroidaler Östrogene und NSAIDs zu untersuchen. Hierbei wurden Proben von Watch List Probenahmestellen aus 14 EU-Mitgliedstaaten und 4 Schweizer Kantonen gesammelt. Diese wurden an die Firma BioDetection Systems (NL) versendet, die Probenvorbereitung und -verteilung durchführte. Ziel war es, sowohl das Vorhandensein wie auch die Aktivität von Östrogenen und NSAIDs durch chemische Analysen (LC-MS/MS) und effektbasierte *in vitro* Methoden in Oberflächengewässern zu quantifizieren. Die detaillierten Ziele dieser Arbeit hierbei waren:

- die Überprüfung eines vom Ökotoxzentrum für Östrogene etablierten Probenahmeprotokolls (SOP) hinsichtlich der Eignung für NSAIDs;
- das Abschätzen möglicher Matrixeffekte;
- das Anpassen und die Optimierung des NSAID *in vitro* Assays hinsichtlich kostengünstigem Screening, maximale Empfindlichkeit und Zuverlässigkeit;
- die Validierung des optimierten NSAID *in vitro* Assays;
- der Vergleich von effektbasierter und chemischer Analysemethode.

Ergebnisse

Änderung des Probenahmeprotokolls

Das für Östrogene entwickelte Standardprotokoll des Ökotoxzentrums konnte nicht für die Probenahme von NSAIDs verwendet werden, da im Rahmen von Vorversuchen NSAID-Instabilitäten in Aluminiumflaschen bei pH 3 beobachtet wurden. Daher musste eine alternative Probennahme entwickelt werden. In Abweichung zur ursprünglich etablierten Methode wurden die Proben in den Aluminiumflaschen nicht angesäuert. Ein Standardverfahren wurde entwickelt, das auf der Festphasenextraktion von 700 mL einer wässrigen Probe basierte. Mittel- und langfristige Stabilitäten bei der Lagerung von NSAID-haltigen Proben in den Aluminiumflaschen wurden untersucht. Dabei wurden mit Ausnahme von Ketoprofen nach 24 Wochen Lagerung für die getesteten NSAIDs Diclofenac, Ibuprofen, Indomethacin und Naproxen eine deutliche Konzentrationsabnahme beobachtet. Daher wurde festgelegt, dass die Wasserproben nach der Probenahme für einen Zeitraum von maximal 4 Wochen bei 4°C gelagert werden dürfen.

Anpassung und Optimierung des NSAID *in vitro* Asssays

Der NSAID *in vitro* Assay wurde so modifiziert, dass eine akzeptable Nachweisgrenze für die Überwachung von Watch List Proben erreicht wurde. Hierbei war das Ziel eine Quantifizierung von NSAID Aktivität im Bereich kleiner 50% der Umweltqualitätsnorm von Diclofenac (0,05 µg/L) zu gewährleisten. Dabei wurde der Test bezüglich Empfindlichkeit und Zuverlässigkeit optimiert. Käufliche Arachidonsäureprodukte wurden auf ihre Eignung als Substrat für die in der NSAID-Sensorzelle exprimierte Cyclooxygenase untersucht. Das Arachidonsäureprodukt A3611 (Sigma-Aldrich, > 98 % Reinheit) führte verglichen mit einem ähnlichen Produkt mit einer Reinheit > 95% (Sigma-Aldrich A10931) im Assay zu einer signifikant höheren Cyclooxygenaseaktivität. Dieses Produkt wurde daher in allen weiteren Experimenten verwendet. Die Autoxidation von Arachidonsäure wurde minimiert, indem reine Arachidonsäure in wasserfreiem mit Stickstoff gesättigtem DMSO gelöst und diese Stammlösung unmittelbar vor Beginn des Assays in HEPES-Puffer verdünnt wurde. Ein Testprotokoll für den optimierten NSAID *in vitro* Assay wurde zusammengestellt.

Validierung des optimierten NSAID *in vitro* Assay

Die Validierung wurde auf das analytische Verfahren zur quantitativen Bestimmung von NSAID Aktivität im Konzentrationsbereich der vorgeschlagenen Umweltqualitätsnorm für Diclofenac ausgerichtet. Die Validierungsmerkmale Linearität, Messbereich, Wiederfindung, Präzision, und Nachweisgrenze wurden bestimmt.

<u>Linearität:</u>

Die Konzentrations-Wirkungskurve des NSAID *in vitro* Assays zeigt einen sigmoiden Inhibitor-typischen Verlauf. Der sigmoide Verlauf der Kurve kann mit Hilfe einer 4-Parameter Funktion mit variabler Steigung simuliert werden. Die Effektkonzentration, bei der eine halbmaximale Hemmung erfolgt (EC50) wurde auf 2,4 µg/L Diclofenac-Equivalenten berechnet.

<u>Messbereich:</u>

Der Messbereich des *in vitro* Assays, bei dem das analytische Verfahren bzw. dessen sigmoide Konzentrations-Wirkungskurve noch ein geeignetes Verhalten für eine Berechnung der Konzentration aufweist, beträgt 1,2 – 7 µg/L Diclofenac. In Folge eines Anreicherungsprozesses der Umweltproben um den Faktor 140 wird eine Empfindlichkeit des Assays erreicht, die für die geforderte Nachweisgrenze benötigt wird.

<u>Wiederfindung:</u>

Nach Zusatz von Diclofenac in Wasserproben wurde die Übereinstimmung der wahren Konzentration mit der gefundenen Konzentration verglichen. Die Wiederfindung zugesetzten

Diclofenacs für drei unterschiedliche Konzentrationen betrug zwischen 95 und 105 %. Diese Genauigkeit wurde bei einer relativen Standardabweichung von 2 – 8 % als ausreichend beurteilt.

Präzision:
Die Wiederholbarkeit der Analyt-Bestimmung einer Probe mit einer Konzentration von 3,6 µg/L Diclofenac wurde in 8 Replikaten mit einer relativen Standardabweichung von 8 % belegt.

Nachweisgrenze:
Die Nachweisgrenze für nicht aufkonzentrierte Proben wurde aus dem Dreifachen der Standardabweichung des Hintergrundsignals auf 0,9 µg/L berechnet. Die Festphasenextraktion wurde so durchgeführt, dass nach Auflösung des Pellets eine Konzentration um den Faktor 140 erreicht wurde. Daraus ergab sich eine Nachweisgrenze für das gesamte analytische Verfahren von 6 ng/L. Damit zeigt der NSAID *in vitro* Assay eine ähnliche Empfindlichkeit wie die chemische Analyse, für die die Bestimmungsgrenze bei 12 ng/L liegt.

NSAID-Aktivität in den Watch List Proben
Aufgrund unsachgemäßer Lagerung durch Dritte und dem damit verbundenen Substanzverlust konnten nur 39 von 71 Oberflächenwasserproben gemessen werden. Diese Watch List Proben wurden zusammen mit 5 Kontrollen auf Diclofenac und anderen Inhibitoren der Cyclooxygenase mittels LC-MS/MS analysiert. Parallel dazu wurden in diesen Proben Diclofenac-Äquivalente mit dem optimierten NSAID *in vitro* Assay bestimmt. Bei den meisten Proben lag das Diclofenac-Äquivalent etwas über der mittels LC-MS/MS bestimmten Diclofenac-Konzentration. Die Cyclooxygenase-Inhibitoren Fenoprofen, Ibuprofen, Indomethacin, Ketoprofen, Naproxen, Paracetamol und Salicylsäure konnten mittels LC-MS/MS nicht in signifikanten Mengen nachgewiesen werden. Daher kommen wir zu dem Schluss, dass die NSAID-Aktivität in den getesteten Watch List Proben überwiegend von Diclofenac verursacht wurde. In 14 von 39 (36%; LC-MS/MS) und 16 von 39 (41%; NSAID *in vitro* Assay) Umweltproben lag der Gehalt an Diclofenac bzw. das Diclofenac-Äquivalent über der angestrebten Umweltqualitätsnorm für Diclofenac von 0,05 µg/L.

Alternative Probenanreicherung
Für die meisten der getesteten NSAID-Verbindungen konnte bei der entwickelten Festphasenextraktion (SPE) eine gute Extraktionseffizienz zwischen 80% und 100% erreicht werden. Oberflächengewässer können jedoch sehr unterschiedliche Matrixkomponenten enthalten, die sich negativ auf die SPE auswirken können. Im Falle der chemischen Analytik kompensieren in solchen Fällen zugesetzte isotopenmarkierte Standards solche Verluste und gewährleisten dadurch trotzdem eine zuverlässige Quantifizierung durch LC-MS/MS. Im Gegensatz zum chemischen Analyseverfahren führt ein Verlust an Analyt während der SPE Konzentrierung zu einer reduzierten absoluten Menge an Analyt, in Folge dessen eine zu geringe Menge an Diclofenac-Äquivalenten mit dem NSAID *in vitro* Assay bestimmt wird. Um einen Verlust von NSAIDs durch eine reduzierte Extraktionseffizienz zu vermeiden und die Messung des gesamten komplexen Gemisches zu ermöglichen, wurde eine alternative Konzentrationsmethode entwickelt. Als Alternative wurde ein Vakuumkonzentrationsverfahren erfolgreich getestet. Oberflächenwasserproben vor und nach der Einleitungsstelle einer Kläranlage sowie der Ablauf dieser Kläranlage wurden mittels LC-MS/MS und dem NSAID *in vitro* Assay analysiert. Alle Analysendaten zeigten eine gute Übereinstimmung. Es wird daher für zukünftige Messungen von Oberflächenwasserproben eine alternative Aufkonzentrierung durch Vakuumkonzentration empfohlen.

Schlussfolgerungen
Der optimierte NSAID *in vitro* Assay erfüllt mit einer Nachweisgrenze von 6 ng/L Diclofenac die

geforderte maximal akzeptable Methodennachweisgrenze für die Überwachung der Watch List Proben. Die Genauigkeit und Präzision sind von ausreichender Qualität. Es wurden hochwertige Überwachungsmessdaten erstellt. Die Anwendbarkeit dieses wirkbasierten Testverfahrens als Ergänzung zur gezielten Überwachung von Einzelsubstanzen in der aquatischen Umwelt gemäß der EU-Wasserrahmenrichtlinie wurde gezeigt. Die optimierte effektbasierte Methode zur Quantifizierung von NSAID Aktivität zeigt eine vergleichbare Sensitivität zur chemischen Analytik und eignet sich gut als Screeningmethode. Chemisch-analytische und effektbasierte Methoden zur Messung von NSAIDs können kombiniert werden wodurch folgende Vorteile beider Methoden genutzt werden können:

- Eine Zuordnung von Wirkungen auf Einzelsubstanzen und die Einschätzung von Mischungseffekten
- Die Untersuchung von Wirkungen komplexer Mischungen
- Die Vermeidung falsch negativer Messungen
- Das Monitoren der NSAID Arzneimittelgruppe in der aquatischen Umwelt

1 Introduction

Due to demographic changes, the consumption rates of pharmaceuticals are expected to dramatically increase in the future. Since most of the drugs are not completely removed by conventional wastewater treatment rising pharmaceutical concentrations are expected to occur in the water cycle (1) (2). In consequence, increasing amounts of compounds are discharged into streams and lakes. The last decade, this topic has become an issue of great public and political concern. Within the EU Water Framework Directive implementation process, the estrogens EE2, E2, the painkiller diclofenac, and other substances had been placed on a watch list for which EU-wide monitoring data must be gathered. Due to the phylogenetic conservation of central signal transduction pathways among vertebrates, highly specific pharmaceuticals affect many vertebrate species, including fish, at low concentrations (3). To assess the risk resulting from exposure of aquatic organisms to a distinct pharmaceutical class, not only effects induced by single parent compounds, but those of all substances characterized by the same mode of action (MOA) including transformation products have to be taken into account. Due to this, effect-directed analysis (EDA) is a suitable tool to assess the overall environmental impact for a class of substances with the same mode of action. The combination of EDAs with chemical analysis reduces the risk to underestimate the toxicity of environmental samples.

During an environmental estrogen monitoring program initiated by the Swiss Centre for Applied Ecotoxicology and the German Environment Agency (UBA), approx. 100 grab samples from Watch List sampling stations in 14 EU member states and 4 Swiss cantons were taken to investigate the applicability of effect-based methods for monitoring steroidal estrogens and to compare their results with chemical analysis. As part of the sampling of surface water from watch list sampling sites planned here, the UBA project (3717 63 440 0) intended to detect diclofenac of respective samples by chemical analysis (LC-MS/MS) and to measure NSAID activity by an effect-based method.

For the quantification of NSAID activity in environmental samples, a fast responding MOA-directed NSAID *in vitro* assay is described using a biosensor cell line expressing appropriate sensor and fluorescent reporter proteins to allow for specific and sensitive inhibition of cyclooxygenase (4). The aim of this project was to investigate the applicability of this NSAID *in vitro* assay for monitoring cyclooxygenase inhibition activity of environmental probes. For this purpose, it was essential to prove the established estrogen sampling protocol and to adapt and optimize the NSAID *in vitro* assay for a cost-effective screening with maximal sensitivity. The NSAID *in vitro* assay was optimized and validated and the practical applicability for the quantification of NSAID activity in surface water was demonstrated. The chemical target analysis of the Watch List samples was compared with the effect-based system in parallel to assess a possible underestimation of the respective environmental impact.

2 Sample collection, storage, and preparation

2.1 Modification of the estrogen sampling protocol for influencing NSAID stability

A standard operation protocol (SOP) for sampling, preparation, and measurement of the estrogens EE2, E2, E1 was developed by the Swiss Centre for Applied Ecotoxicology (Eawag, Dübendorf Switzerland) and partners during a preceding estrogen monitoring project. To prevent microbial degradation and ensure sufficient stability of the hormones present in low picomolar concentrations, samples were acidified to pH 3.0 and stored in aluminum bottles prior to chemical analysis.

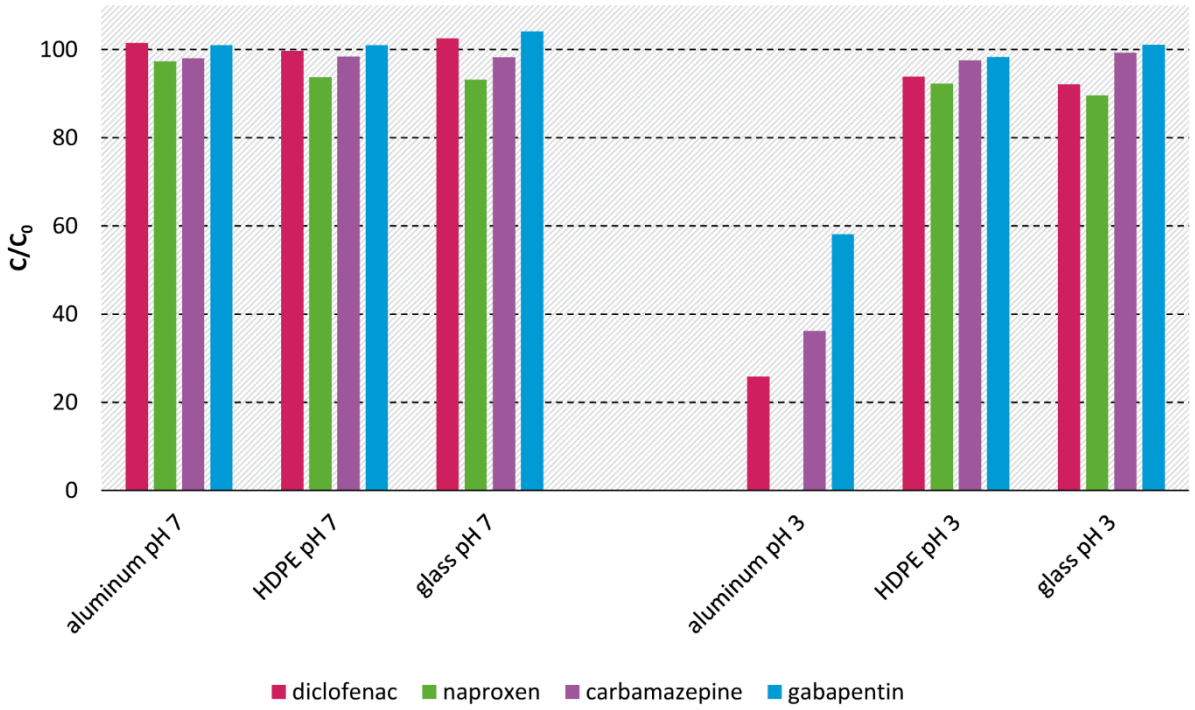
It was essential to prove the established estrogen sampling protocol for its suitability for NSAID sampling. First preliminary tests addressed uncertainties regarding the stability of the NSAID compounds diclofenac and naproxen during transport and storage in aluminum bottles at pH 3.

For the first stability test, surface water from the River Main was sampled and fortified with 100 ng/L of all analytes. First analyses were performed right after fortification and after a storage time of one week in the freezer and at 4 °C in a cooling room. Surprisingly, significantly lower concentrations were measured after 7 days for all analytes (A.1), which identified the existing SOP to be inappropriate for NSAIDs.

In order to prove whether the bottle material or the low pH led to the reduced concentrations of NSAIDs, the test was repeated with fresh and non-spiked surface water. Three different types of bottles (aluminum, glass, and high-density polyethylene (HDPE) were used and two different pH values (pH 3 and pH 7) were tested. As shown in Figure 1, the combination of sample acidification and storage in aluminum bottles had a pronounced effect on the NSAID stabilities and led to significantly reduced concentrations. While the cox inhibitor naproxen was no longer detectable above the limit of detection after one week of storage, only less than 30% of the initial amount of diclofenac was detectable when stored under this condition. In addition to NSAIDs, this reduction was also observed for two other pharmaceuticals carbamazepine and gabapentin. For every other combination of bottle material and pH, the concentrations measured at day 7 were similar to those on day 0. These results clearly indicate that the aluminum surface in acidic conditions somehow catalyzes degradation of the tested pharmaceuticals. **Consequently, the established sampling protocol for estrogens could not be applied for the sampling of NSAIDs**. Therefore, an alternative sampling method had to be developed.

Based on these results it was decided to use aluminum bottles for sampling without acidification. A respective sampling instruction was developed and forwarded to the project partner.

Figure 1: c/c_0 of four different micropollutants.



Surface water was analyzed at the day of sampling and after one-week storage time at 4°C in different types of bottles at pH 3 and pH 7.
C_0 = concentration at the day of sampling; C = concentration after one-week storage

reference: DVGW-Technologiezentrum Wasser

2.2 Development of a sampling protocol and an analytical method for NSAIDs

A standard operating procedure based on the solid-phase extraction (SPE) of 700 mL of an aqueous sample was established at the TZW. To ensure that the sample volume does not negatively affect the extraction efficiency, preliminary tests were performed by the extraction of 700 mL drinking water and surface water.

700 mL water samples were adjusted to pH 3 with hydrochloric acid and the analytes were pre-concentrated with a polymeric sorbent material (Strata-X, 200 mg, Phenomenex, Aschaffenburg, Germany). Prior to enrichment, the solid phase extraction (SPE) cartridges were washed with 2x3 mL methanol and 2x3 mL distilled water (pH 3). After SPE the sorbent was dried under a gentle stream of nitrogen and eluted with 2x2.5 mL methanol and 2.5 mL acetone. The eluate was blown down to dryness with nitrogen and the dry residue was provided for further analyses.

In order to only assess the extraction efficiency and to exclude matrix effects, like a signal suppression in the interface of the mass spectrometer, one subset of samples was spiked prior to SPE and compared with samples spiked in the eluate after SPE (also including matrix).

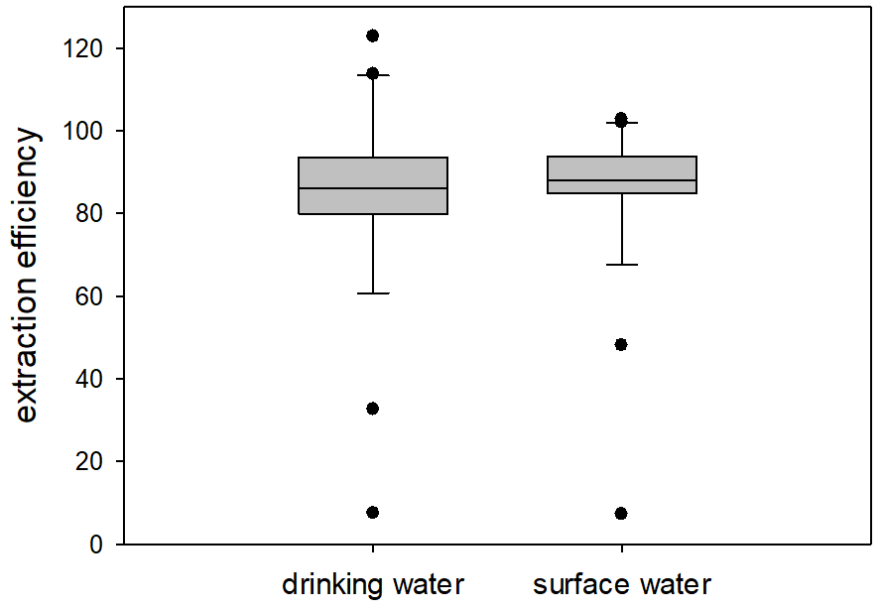
To achieve hydrophobic interactions between the target molecules and the sorbent material the samples were adjusted to pH 3. For example, diclofenac occurs in its anionic form at neutral pH and has a logP value of around 1.90. When the pH is decreased to pH 3, the molecule is mostly

uncharged and the logD value increases to around 4.5 which allows for better extraction during sample pretreatment. The following compounds, many of which are present in environmental samples, were tested: bezafibrate, carbamazepine, clofibric acid, diazepam, diclofenac, etofibrate, fenofibrate, fenofibric acid, fenoprofen, gemfibrozil, ibuprofen, indometacin, ketoprofen, naproxen, pentoxifylline, phenacetin, gabapentin, paracetamol, primidone, 10,11-dihydro-10,11-dihydroxycarbamazepine, salicylic acid. For the majority of these compounds, a good extraction efficiency between 80% and 100% could be achieved (Figure 2). In both box plots, the two outliers which were poorly extracted are the highly polar compounds gabapentin and paracetamol (also known as acetaminophen). Even at pH 3 both have rather low logD values of 0.38 and 0.65, respectively (calculated with Percepta/ACD-Labs, Release 2016.2). Although showing low extraction efficiencies both compounds were kept in the analytical method. In such cases, isotopically labeled internal standards or a matrix matched calibration can compensate for losses and still ensure a reliable quantification if the extracted absolute amount still enables to detect the compound with a sufficient signal-to-noise ratio.

Importance of pH for solid-phase extraction

The most commonly used measure of lipophilicity is LogP, this is the partition coefficient of a molecule between an aqueous and lipophilic phase. The higher the lipophilicity, the higher the retention on the SPE material should be. However, the majority of known drugs contain ionizable groups and are likely to be charged at neutral pH. Thus, the LogP only correctly describes the partition coefficient of neutral (uncharged) molecules. LogD instead refers to the LogP at a specific pH. For ionizable compounds (acids and bases), LogD can be altered by pH because the distribution of charged and uncharged forms would change, and the uncharged form is more hydrophobic.

Figure 2: Extraction efficiency of all target analytes from drinking water and surface water.



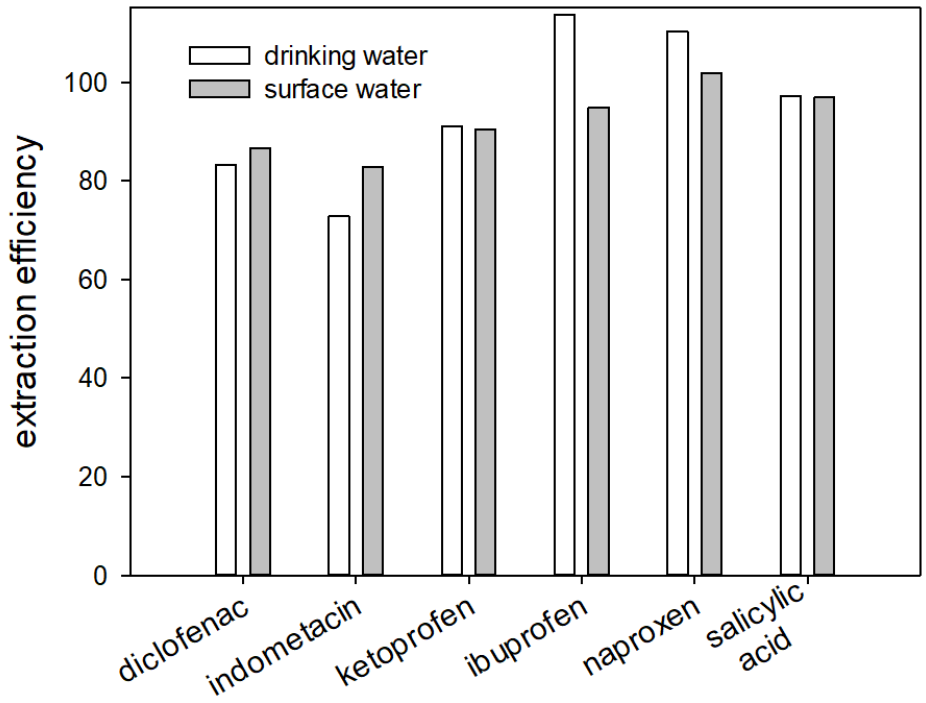
Extraction volume 700 mL. Compounds with poor extraction efficiency in drinking water and surface water were gabapentin and paracetamol. Upper and lower limit of the box spans the first quartile to the third quartile, line in the box represents the median value, whiskers indicate the 5 and 95 percentiles with every outlier plotted as individual point.

reference: DVGW-Technologiezentrum Wasser

Some pharmaceuticals were included in the chemical analysis although they do not inhibit cyclooxygenase (COX). The COX inhibition of paracetamol is still controversially discussed (5) but generally the compound is considered to weakly inhibit COX-1 (6) and to have no effect on COX-2 (7).

For all non-selective COX inhibitors the extraction efficiencies were satisfying and it can be concluded that the existing method can be applied with the sample volume of 700 mL. As Figure 3 shows the extraction efficiencies for the key component diclofenac (and others) are slightly below 100%. During chemical analysis, the application of suitable isotopically labeled internal standards will compensate for extraction efficiencies different from 100% as they behave like the non-labeled compounds. However, it has to be taken into account that the absolute amount provided for the bioassays could be reduced if the extraction efficiency is below 100%. Surface waters can contain very different matrix components which can affect SPE but it is beyond the scope of this project to test the extraction efficiency for every surface water sample to be analyzed within the project. It needs to be discussed how this aspect is taken into account when interpreting the results, e.g. by interpreting results from the COX assay as "minimal concentrations".

Figure 3: Extraction efficiencies of COX inhibitor target analytes from drinking water and surface water. Extraction volume was 700 mL.



reference: DVGW-Technologiezentrum Wasser

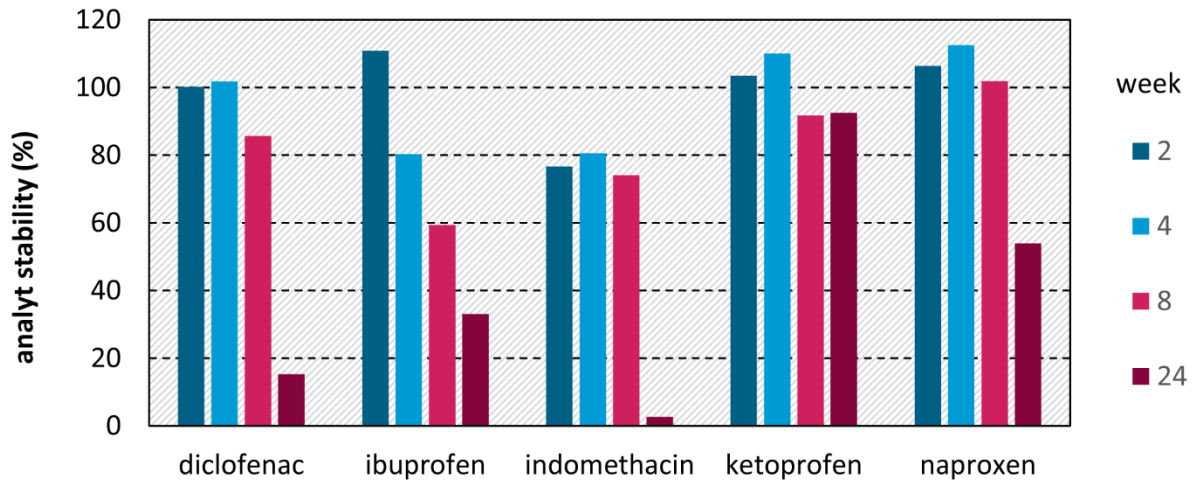
2.3 Mid- and long-term stability of NSAIDs in aluminum bottles

The sampling of the surface water samples took place in several European countries. For the collection, processing, and organization of the sample shipment, a certain time was required until the samples could be analyzed in the laboratory. Therefore, it was important to know the stability of the compounds to be analyzed which were stored in aluminum bottles. To assess mid- and long-term stability of the analytes, water from the River Main was sampled and spiked

with 300 ng/L respectively of the analytes diclofenac, ibuprofen, indomethacin, ketoprofen, and naproxen. After storage times of 2, 4, 8 and 24 weeks at 4°C analyses were performed. It was found that up to two weeks of storage at 4° diclofenac, ibuprofen, ketoprofen, and naproxen were stable without any degradation. 80 % of indomethacin could be found after 2 weeks of storage. With the exception of ketoprofen, a noticeable loss was observed after 24 weeks of storage for all analytes Figure 4.

It was concluded that the environmental probes should be stored for maximal 4 weeks at 4 °C before analysis.

Figure 4: Stability of analytes during storage in aluminum bottles at 4°C



reference: DVGW-Technologiezentrum Wasser

3 Effect directed NSAID analysis

Before starting with the measurement of Watch List probes of several different sampling sites it was essential to adapt and optimize the NSAID *in vitro* assay for a screening with maximal sensitivity. The effect-based assay had to fulfill the required maximum acceptable method detection limit for the watch list monitoring being < 50% of the envisaged environmental quality standard of 0.05 µg/L diclofenac. Due to the self-fluorescence of some matrices, the signal-to-noise ratio had to be optimized. Arachidonic acid is sensitive to oxygen and therefore, buffer conditions had to be found allowing sufficient stability of arachidonic acid in the stock solution as well as during the execution of the test. Before measuring the Watch List samples, a validation of the optimized NSAID *in vitro* assay was performed to ensure sufficient accuracy and precision.

3.1 Matrix effects

SPE extracts from 700 mL surface water were prepared by TZW according to 2.2. Samples were fortified with diclofenac prior to solid-phase extraction. During first evaluations, it appeared that the extracts showed a considerable self-fluorescence leading to a decreased signal-to-noise ratio compared to wastewater treatment plant effluents (4). The signal-to-noise ratio was optimized by increasing the arachidonic acid concentration in the NSAID *in vitro* assay. Arachidonic acid is poorly soluble in aqueous buffer. Therefore, we increased the DSMO concentration. The higher arachidonic acid concentration in the assay resulted in a higher glutathione oxidation rate and the concomitant more intensive redox sensor signal. **Best signal-to-noise-ratio was reached starting the assay with a final concentration of 400 µM arachidonic acid in a buffer containing 1 % DMSO.**

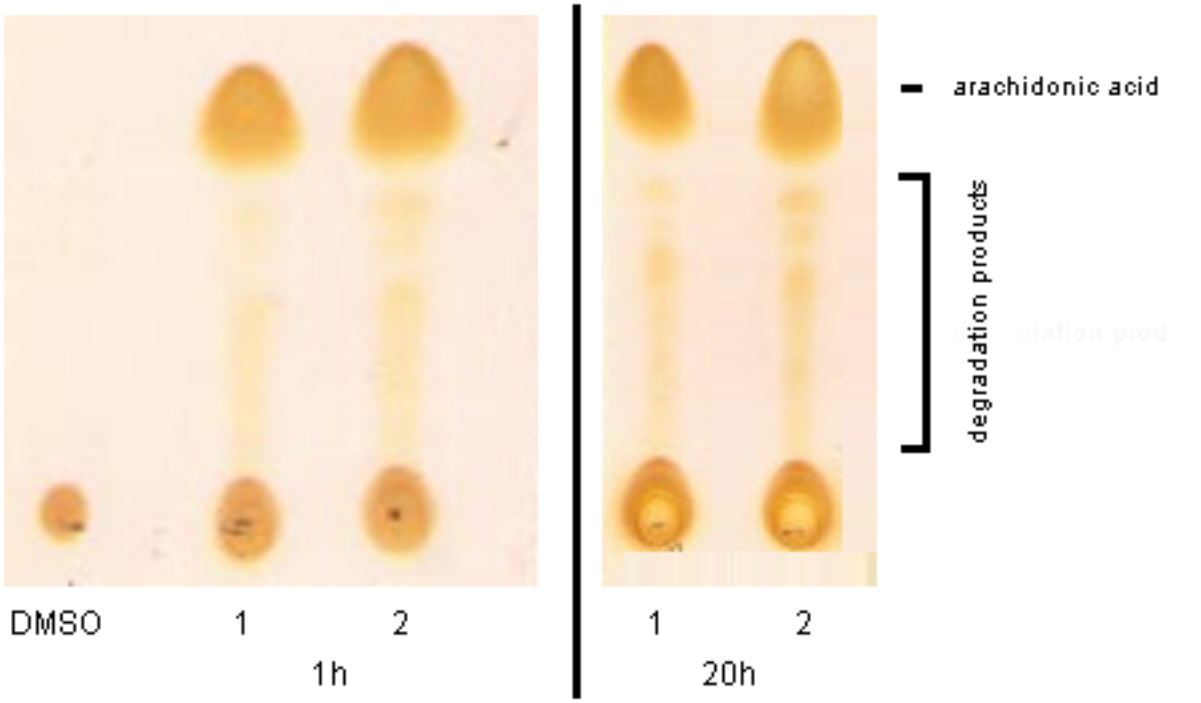
3.2 Stability of arachidonic acid

Arachidonic acid is a polyunsaturated fatty acid which undergoes autoxidation in the presence of oxygen leading to a complex mixture of prostaglandin-like structures (8). Thus, the stability of arachidonic acid in oxygen-containing buffer solutions is limited.

Arachidonic acid autoxidation in stock solutions
For assessing the stability of an arachidonic acid stock solution, 170 mM arachidonic acid was dissolved in DMSO. The arachidonic acid solution was stored at room temperature for 1 h and 20 h under air atmosphere. The thin-layer analysis (Macherey-Nagel, Polygram SIL G/UV254) was performed after 5-time dilution of the arachidonic acid stock solution in methyl tert-butyl ether (MTBE). The chromatographic fluid was cyclohexan:MTBE (7:3). The staining of arachidonic acid was achieved by reaction with iodine. Two commercially available arachidonic acid samples (Sigma Aldrich A3611 and 10931) were analyzed. After 20 h storage at room temperature, there was only a slight increase in degradation products visible compared to 1 h storage (Figure 5). It was concluded that the purity and stability of both arachidonic acid preparations were comparable.

Although both arachidonic acid preparations showed comparable weak degradation products in the thin-layer chromatography, the COX-1 specific signal differed significantly in the NSAID *in vitro* bioassay. The arachidonic acid sample A3611 showed a significant higher cyclooxygenase substrate activity compared to the similar product A10931. **Therefore, the arachidonic acid product A3611 was used in all further experiments.**

Figure 5: Thin-layer chromatography of arachidonic acid stock solutions



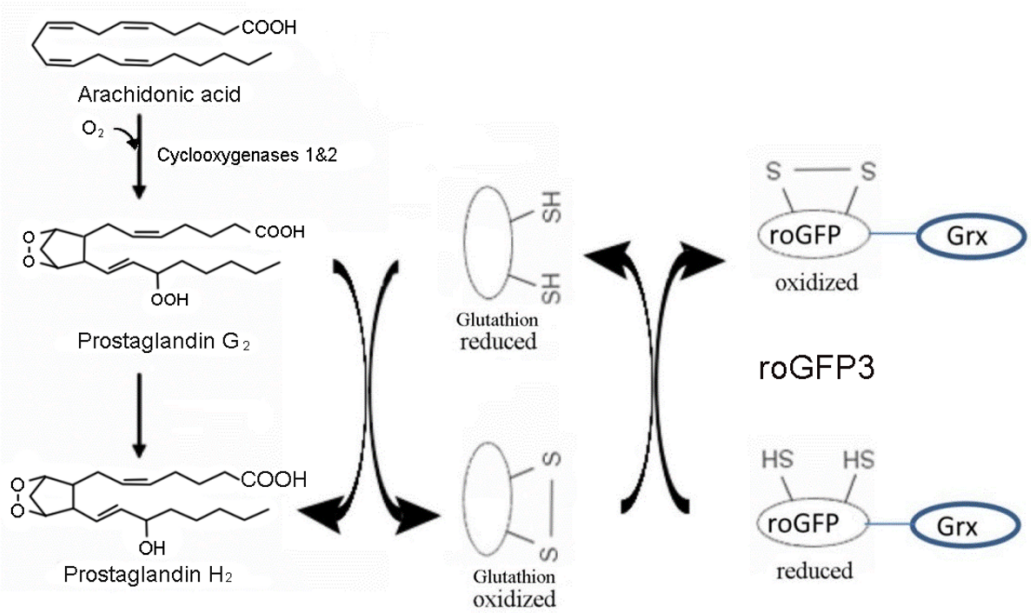
The arachidonic acid sample A3611 (1; Sigma-Aldrich, > 98 % purity) and the sample A10931 (2; Sigma-Aldrich, > 95% purity) showed similar degradation products after incubation at room temperature for 1 and 20 h.

reference: SIZ Zellkulturtechnik

Arachidonic acid autoxidation during the NSAID *in vitro* assay

The NSAID biosensor cell line expresses the genetically encoded fluorescent redox sensor roGFP3 together with the human COX-1 after induction. In the presence of the COX-1 substrate arachidonic acid, accumulation of reactive oxygen species oxidizes the roGFP3 redox sensor leading to an increase in the ratio of emissions at 528 nm when excited at 395 nm and 485 nm in the cells (Figure 6). After pre-incubation of the sensor cells with the COX-1 inhibitor diclofenac prior to the addition of arachidonic acid, this ratio increase was reduced. However, the addition of arachidonic acid resulted in COX-independent oxidation of the redox sensor most likely due to arachidonic acid autoxidation products. Even maximum inhibition of COX-1 resulted in arachidonic acid-dependent oxidation of the redox sensor. Preincubation with 100 µM diclofenac led to a partial redox sensor oxidation (Figure 7, ochre curve) and was also observed in cells expressing the redox sensor without COX-1. This COX-independent redox sensor signals varied using different arachidonic acid stock solutions. The COX-1 specific signal-to-noise ratio resulting from the maximum signal (without inhibition) over the minimum signal (maximal inhibition) was different for several sample aliquots. We concluded that this results from autoxidation during storage.

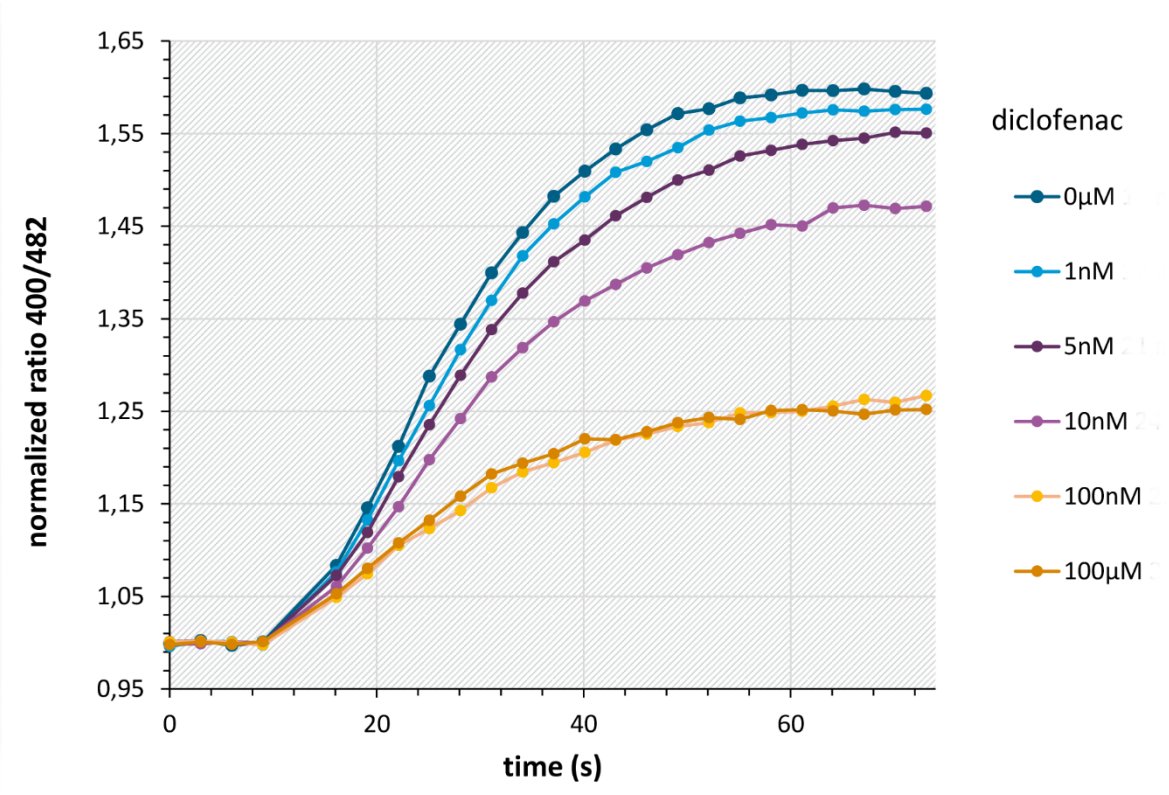
Figure 6: COX-1 dependent oxidation of the redox sensor



During the COX-catalyzed reaction of the substrate arachidonic acid reactive intermediate lipid peroxides are formed that can be detected via the change of fluorescence properties of the roGFP3 redox sensor.

reference: SIZ Zellkulturtechnik

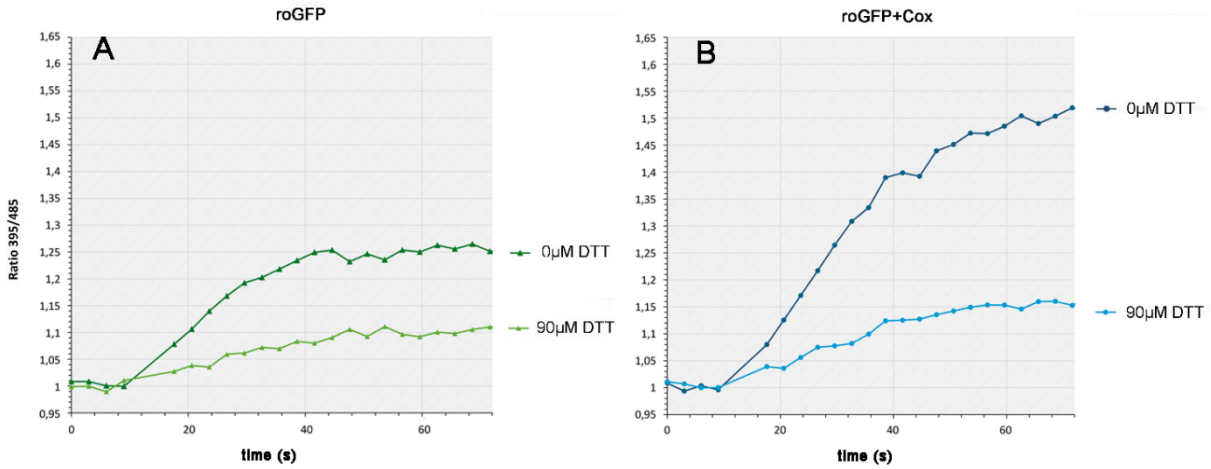
Figure 7: Inhibition of the COX-1 catalyzed oxidation of arachidonic acid



reference: SIZ Zellkulturtechnik

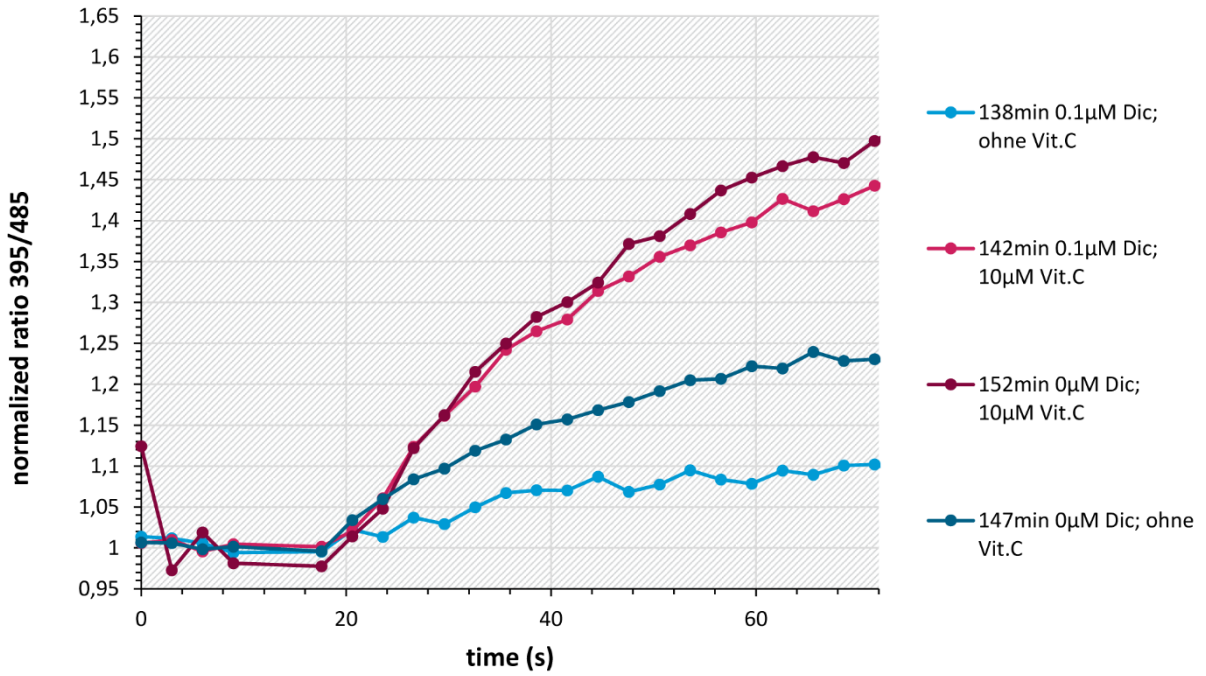
For the possible reduction of autoxidation and better stability of arachidonic acid, antioxidants were added to arachidonic acid aqueous solutions. The addition of DTT to a final concentration of 90µM resulted in a reduction of the COX independent oxidation of the redox sensor due to a reduction of autoxidation products of arachidonic acid. Cells expressing the redox sensor without COX showed a significant reduction of the fluorescence ratio after arachidonic acid addition (Figure 8 A). Under these conditions, however, the COX-dependent oxidation of the redox sensor is also reduced (Figure 8 B). **Therefore, DTT is not useful, as this leads to a reduced signal-to-noise ratio.**

Figure 8: Reduction of the redox sensor signal by DTT



reference: SIZ Zellkulturtechnik

Figure 9: Enhancement of the redox sensor signal by vitamin C



reference: SIZ Zellkulturtechnik

In contrast to DTT, the addition of vitamin C to the arachidonic acid aqueous solution resulted in an increase of the oxidation of the redox sensor. This oxidative effect was unexpected because vitamin C has antioxidant properties. Under the experimental conditions, vitamin C acts as an oxidant resulting in the generation of reactive oxidative species which are able to transfer the cell membrane leading to an oxidation of the redox sensor. The generation of reactive oxygen species was dependent on arachidonic acid. Without arachidonic acid, no oxidation of the redox sensor was observed when vitamin C was added to the sensor cells. This oxidative stress-mediated by vitamin C and arachidonic acid was COX independent (Figure 9).

The addition of antioxidants like DTT or vitamin C to arachidonic acid-containing solutions resulted in a decrease of the signal-to-noise ratio and therefore could not be used in the assay.

We therefore dissolved arachidonic acid in water-free DMSO under nitrogen atmosphere. This resulted in arachidonic acid stock solutions which showed to be more stable when stored in aliquots at -80°C. Diluting this stock solution in HEPES buffer immediately before starting the assay resulted in best signal-to-noise ratios (Figure 10).

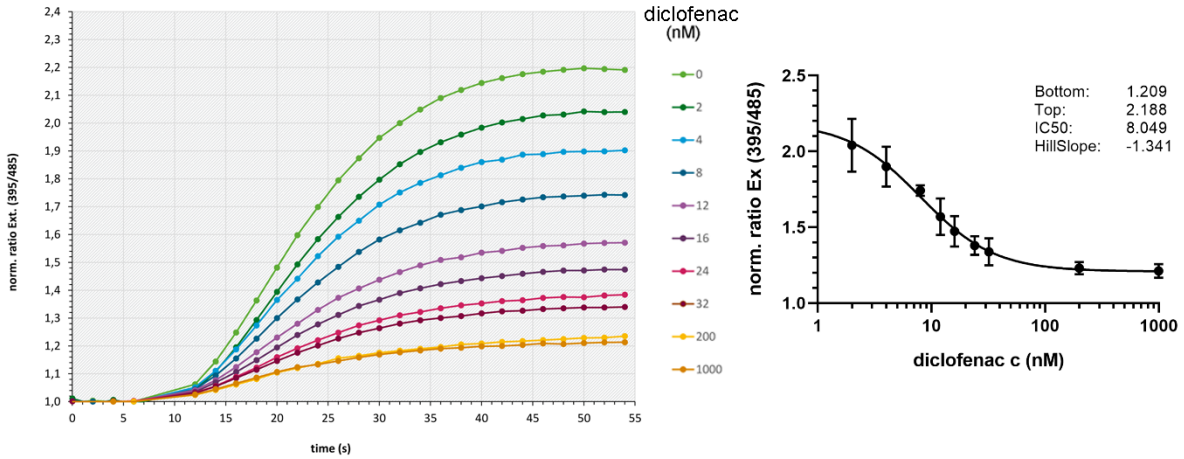
3.3 Optimization and validation *of the* NSAID *in vitro* assay

3.3.1 Optimization of the NSAID *in vitro* assay

NSAID sensor cells expressing the redox sensor and COX-1 showed some slight variation in the initial redox state of the sensor. During optimization of the assay, this difference was minimized by incubation of the sensor cells in a buffer containing 1 µM DTT. Arachidonic acid solutions were prepared according to 3.1 and 3.2.

The optimized NSAID *in vitro* assay was performed as follows.
Diclofenac standard solutions were prepared in HEPES buffer with DTT (25 mM HEPES pH 7.4, 120 mM NaCl, 6 mM $NaHCO_3$, 5.5 mM D-glucose, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.4 mM KCl, 1% DMSO, 1 µM DTT). The biosensor cell line expressing the genetically encoded fluorescent redox sensor roGFP3 together with the human COX-1 after induction was seeded 24 h before the assay in 96-wells in Ham's F12 medium (10% FCS). Induction of expression followed the withdrawal of doxycycline. Cells were pre-incubated with 60 µl diclofenac or the diluted extracts in HEPES buffer for 1 h prior to the start of the assay. The Arachidonic acid stock was dissolved in DMSO saturated with nitrogen. Bevor starting the assay arachidonic acid was diluted in HEPES buffer without DTT (25 mM HEPES pH 7.4, 120 mM NaCl, 6 mM $NaHCO_3$, 5.5 mM D-glucose, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.4 mM KCl, 1% DMSO) resulting in a suspension containing 700 µM arachidonic acid. Assay started after addition of arachidonic acid in HEPES buffer without DTT to a final concentration of 400 µM. Measurement of fluorescence intensity ratio continued for 1 min in well mode. Fluorescence measurements were done with a ClarioStar plate reader (BMG Labtech, Ortenberg, Germany). ClarioStar instrument settings for the roGFP3 assay were: excitation: 395 nm and 485 nm; emission 528 nm; well mode. For the measurement of NSAID concentration-response curves ratio 395/485 nm was determined after reaching a plateau. The mean of five measurements was calculated. The mean of the last three values of the response curve Figure 10 left) was used for the concentration-response curve (Figure 10 right). EC50 values were calculated by fitting of the concentration-response curve (variable slope 4 parameter sigmoidal fitting, Graph Pad Prism).

Figure 10: COX inhibition biosensor assay



After the addition of the COX substrate arachidonic acid the oxidized redox sensor generated by oxidized glutathione was determined by measuring the increase in the excitation fluorescence ratio Ex (395 nm/485 nm) emitted at 528 nm. Molarities correspond to the following diclofenac concentrations: 2 nM (0.6 µg/L), 4 nM (1.2 µg/L), 8 nM (2.4 µg/L), 12 nM (3.6 µg/L), 16 nM (4.7 µg/L), 24 nM (7.1 µg/L), 32 nM (9.5 µg/L), 200 nM (59.2 µg/L), 1000 nM (296 µg/L) Normalized ratios (Ex 395/485) were measured in hexaplicates.

reference: SIZ Zellkulturtechnik

3.3.2 Validation of the NSAID in vitro assay

To ensure sufficient accuracy and precision validation of the optimized NSAID *in vitro* assay was performed. The validation was directed to the analytical procedure to quantitatively test cyclooxygenase inhibition activity of NSAIDs fulfilling the required maximum acceptable method detection limit for the watch list monitoring. The validation characteristics linearity, range, accuracy, precision, and limit of detection were evaluated.

3.3.2.1 Linearity

The concentration response curve of the COX inhibition biosensor assay shows a common symmetrical shape. The curve was fitted using a variable slope 4 parameter sigmoidal fitting (Graph Pad Prism). The 4-parameter sigmoidal fitting. The mean, standard deviation, relative standard deviation, coefficient of determination and EC50 were calculated using hexaplicates (Table 1). The top, bottom, Hill Slope and EC 50 values were shown in Figure 10. The EC50 is the concentration of the COX inhibitor that gives a response half between bottom and top and was calculated to be 8 nM (2.4 µg/L).

Table 1: **Linearity**

Concentration (µg/L)	Mean (normalized ratio)	standard deviation	Relative standard deviation
0	2.19	0.14	6.2 %
8	1.74	0.04	2.1 %
16	1.47	0.10	6.8 %
32	1.34	0.09	6.7 %
Goodness of fit (r^2) = 0.923		EC50: 8 nM (2.4 µg/L)	

3.3.2.2 Range

The interval between the upper and lower concentration of the analyte diclofenac for which the analytical procedure has a suitable level of linearity assess the range of the method. **Data obtained during the linearity study estimated the range of the method without a prior concentration process to be 4 – 30 nM (1.2 – 7 µg/L).**

3.3.2.3 Accuracy

The closeness of agreement between the value accepted as a true value and the value found was determined by spiking diclofenac into samples. Spiked samples were prepared at three concentrations over the assay range. Samples were measured in triplicate. For each sample, the theoretical value, assay value, and percent recovery are reported together with the calculated relative standard deviation (Table 2).

The recovery of spiked diclofenac for three concentrations varied between 95 and 105 %. The accuracy was proved to be sufficient with a relative standard deviation of 2 – 8 %.

Table 2: Accuracy

Sample	Amount of standard (µg/L) spiked	found	Relative standard deviation	Recovery
1	2.37	2.26	2.06%	95.3%
2	3.55	3.74	7.65%	105.2%
3	4.74	4.95	6.81%	104.4%

3.3.2.4 Precision - Repeatability

One sample solution containing 12 nM (3.6 µg/L) diclofenac was prepared and 8 replicates were made from this sample solution according to the final method procedure (3.3). Table 3 lists the calculated mean, standard deviation and relative standard deviation.

The repeatability was demonstrated showing a relative standard deviation of 8%.

Table 3: Repeatability

Sample	Normalized ratio
Replicate 1	1.66
Replicate 2	1.62
Replicate 3	1.60
Replicate 4	1.57
Replicate 5	1.62
Replicate 6	1.40
Replicate 7	1.37
Replicate 8	1.71
Mean	**1.57**
Standard deviation	**0.12**
Relative standard deviation	**7.65%**

3.3.2.5 Intermediate Precision

Intermediate precision (within-laboratory variation) was demonstrated by two analysts measuring two concentrations of diclofenac containing samples on different days (Table 4). The samples were first concentrated by SPE, then dissolved in HEPES buffer and measured in various dilutions, reflecting the entire analysis process.

Table 4: Intermediate Precision

Sample	S 1 (µg/L)	S 2 (µg/L)
Operator 1, day 1	0.094	1.282
Operator 1, day 2	0.085	0.629
Operator 2, day 3	0.106	0.833
Mean	0.095	0.915
Standard deviation	0.010	0.334
Relative standard deviation	11%	37%

3.3.2.6 Limit of Detection

The lowest concentration of the standard solution was determined by sequentially diluting the sample. Eight replicates were made from 4 nM (1.2 µg/L) diclofenac solution. The noise signal was determined by measuring a sample without diclofenac in decaplicate. Replicates, standard deviation, relative standard deviation and the mean of the noise signal were shown in Table 5. The limit of detection was calculated from the triple of the noise signal standard deviation. The mean of the noise signal minus three times of the noise signal standard deviation results in a

ratio of 2.0 that could be extrapolated into a concentration of 2.9 nM or 0.86 µg/L diclofenac which is the limit of detection of the NSAID *in vitro* biosensor cell-based assay without a prior concentration of the samples.

The solid-phase extraction was performed in such a way that after the resolution of the pellet a concentration by a factor of 140 was achieved. This resulted in a limit of detection for the entire analytical procedure of 6 ng/L. The NSAID *in vitro* assay shows a similar sensitivity as to chemical analysis with a limit of quantification of 12 ng/L.

Table 5: Limit of Detection

Sample	Normalized ratio
Replicate 1	2.05
Replicate 2	2.08
Replicate 3	1.94
Replicate 4	1.73
Replicate 5	1.82
Replicate 6	1.72
Replicate 7	1.94
Replicate 8	1.92
Mean	**1.90**
Standard deviation	**0.13**
Relative standard deviation	**6,9%**
Noise signal mean	**2.24**
Noise signal standard deviation	**0.08**

3.4 NSAID activity in the Watch List samples

A total number of 71 surface water samples (~5.5 L each) were collected from Watch List sampling stations in 14 EU member states and 4 Swiss cantons between autumn 2017 to spring 2018 and shipped directly to BioDetection Systems (NL), where sample preparation and distribution were performed. Due to improper storage and the resulting loss of substance, only 39 samples could be measured. Samples and 5 field blanks were analyzed for diclofenac along with other pharmaceuticals (COX inhibitors, e.g. ibuprofen) by LC-MS/MS using the method described in (4) and screened *in vitro* for COX inhibition according to 3.3.

For the analysis in the *NSAID in vitro* bioassay, a maximum of 700 mL of the surface water samples was concentrated by solid-phase extraction according to the method described in 2.2. The dry residues were then diluted in HEPES buffer with DTT according to the method described in 3.3.

For the measurement of NSAID activities the concentration-response curve ratio 395/485 nm was determined after reaching a plateau. The mean of five measurements was calculated. For

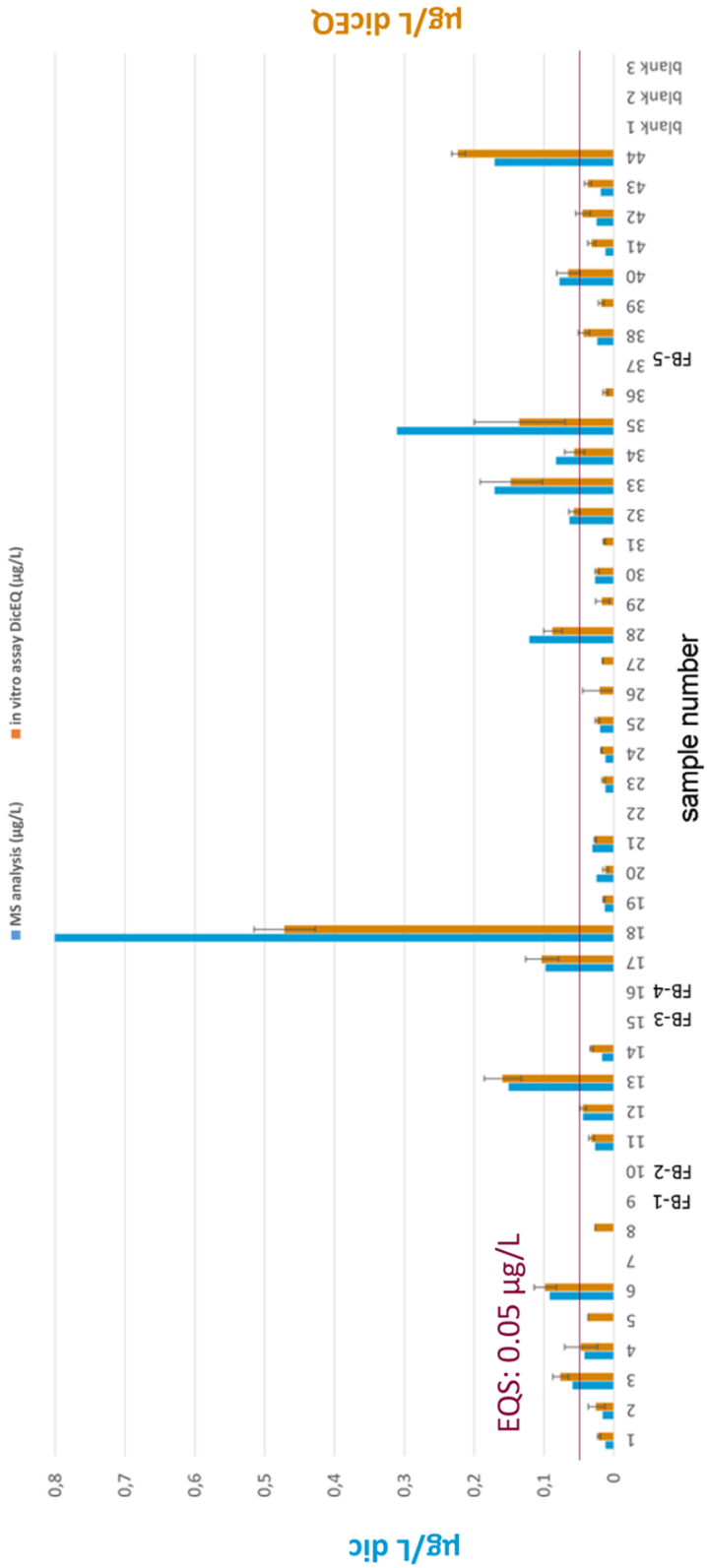
these measurements, the arachidonic acid stock solution was prepared immediately before the measurement.

The chemical analytical and effect-based methods fulfilled the required maximum acceptable method detection limit for the watch list monitoring. Both methods showed similar sensitivity.

As shown in Figure 11 both methods determined the similar amount of diclofenac activity in nearly all of the measured samples (A.2, A.3). For most of the environmental samples measured, the dicEQ was slightly higher than the diclofenac concentration measured by LC-MS/MS. The COX-inhibitors fenoprofen, ibuprofen, indomethacin, ketoprofen, naproxen, paracetamol, and salicylic acid could not be detected in significant quantities by LC-MS/MS. Therefore, we concluded that **the NSAID activity of the environmental samples tested was mainly caused by diclofenac. Twelve out of thirty-nine (31%; LC-MS/MS) and sixteen out of thirty-nine (41%; NSAID *in vitro* bioassay) environmental probes exceeded the environmental quality standard[2]** (9) **for diclofenac.**

[2] Draft EQS Datasheet, Environmental Quality Standard Diclofenac German Environment Agency (UBA), 2017

Figure 11: NSAID analysis of environmental samples



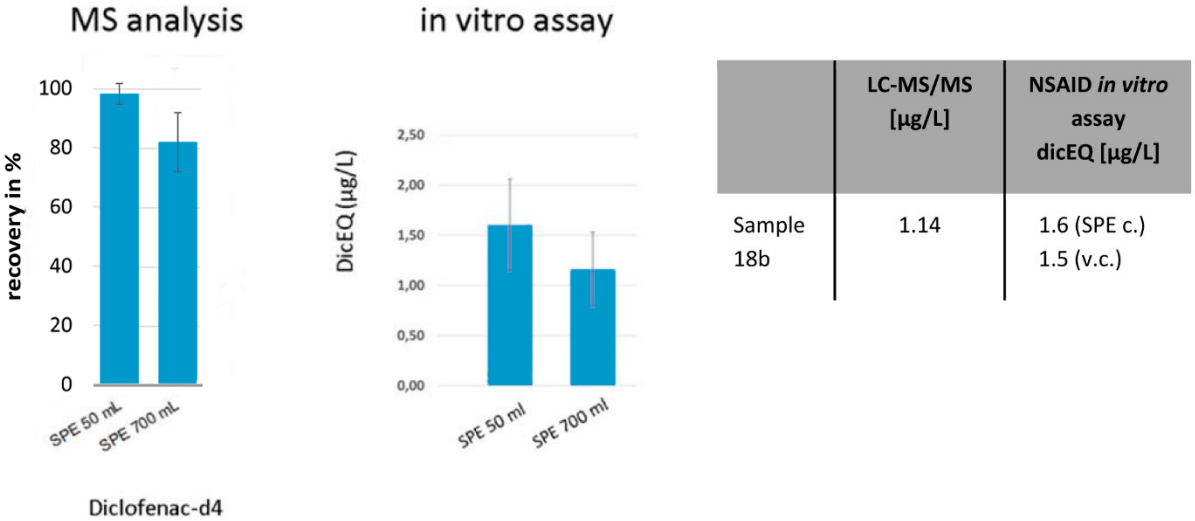
39 samples surface water samples were collected from Watch List sampling stations and analyzed by chemical analysis (LC-MS/MS, blue) and by the NSAID in vitro assay (dark ochre, with indicated standard deviation).
EQS = Environmental Quality Standard
FB = field blank; blanc = distilled water

Sample 18 contained a particularly high organic load due to a nearby sewage treatment plant effluent. Therefore, it was suspected that this high organic load resulted in competition with the binding of diclofenac to the SPE matrix. Therefore, a second sample from this watch list sampling location (sample 18b) was collected and assayed again. 700 mL and 50 mL surface water were concentrated by SPE for this purpose. The SPE concentration of the 700 mL sample showed a recovery of around 80% only. **The SPE concentration of 700 mL Watch List surface water sample 18b resulted in a loss of diclofenac due to overloading the column. 50 mL of the sample was concentrated without loss of substance.**

In contrast to the 700 mL sample no loss of diclofenac-d4 was observed during concentrating the 50 mL sample (Figure 12, MS analysis).

The diclofenac equivalent (dicEQ) for sample 18b measured by the NSAID *in vitro* assay was 1.6 µg/L and higher than the value measured by chemical analyses (1.1 µg/L) (Figure 12). **The higher NSAID activity of the sample 18b is most likely due to mixing effects and / or additional compounds with COX-inhibition activity.**

Figure 12: Volume dependent SPE concentration yield



	LC-MS/MS [µg/L]	NSAID *in vitro* assay dicEQ [µg/L]
Sample 18b	1.14	1.6 (SPE c.) 1.5 (v.c.)

18b samples were spiked with the isomer diclofenac-d4 before and after SPE concentration. We referred the concentration of diclofenac-d4 spiked beore SPE to the concentration spiked after SPE resulting in a recovery < 100% if there is a loss of diclofenac due to uncomplete binding to the column. The SPE concentration of 700 mL surface water sample resulted in a loss of diclofenac due to overloading the column. 50 mL of the sample was concentrated without loss of substance.

reference: SIZ Zellkulturtechnik

3.5 Alternative sample processing

For the majority of tested NSAID compounds, a good extraction efficiency between 80% and 100% could be achieved by the developed solid-phase extraction (2.2). However, surface waters can contain very different matrix components, which can affect SPE. In such cases, isotopically labeled internal standards compensate for losses and still ensure a reliable quantification by LC-MS/MS. In contrast to the chemical analysis procedure, reduced extraction efficiency during SPE leads to a reduced absolute dicEQ amount measured by the NSAID *in vitro* assay.

The NSAIDs contain ionizable groups and are likely to be charged at neutral pH. Therefore, the surface water probes had to be acidified to a pH of 3.0 before SPE resulting in uncharged compounds that are more hydrophobic. This procedure results in the polar or charged compounds flowing through the SPE column.

In order to avoid a loss of NSAIDs due to reduced extraction efficiency and to ensure the measurement of the complete complex mixture, an alternative concentration method was developed. As an alternative to sample concentration by SPE we tested the concentration of water samples by vacuum concentration.

50 mL of Watch List sample 18b was concentrated using a RVC 2-33 CDplus vacuum concentrator (Christ, Germany). The resulting residue was dissolved in 1 mL diluted HEPES buffer in such a way that the resulting osmolality was in a range between 260 to 320 mOSM/kg. The dicEQ determined by the NSAID *in vitro* assay with previous SPE concentration as well as prior vacuum concentration and the LC-MS/MS of the SPE concentrated sample correlated well (Figure 12).

In addition, we collected three water samples one before (S_{before}) one after (S_{after}) a sewage treatment plant discharge and one directly from the sewage treatment plant effluent ($S_{effluent}$). This municipal waste-water treatment plant with a capacity of 130000 population equivalents is located in a region with almost no industry in the surrounding area. 50 mL of the samples were concentrated by vacuum concentration. Samples were measured by the NSAID *in vitro* assay and by LC-MS/MS analytical determination. The dicEQs determined by the NSAID *in vitro* assay and the LC-MS/MS determination of diclofenac correlated well (Table 6) meaning that the NSAID activity is almost exclusively attributable to diclofenac.

Therefore, for the measurement of dicEQs we recommend a concentration procedure of surface water samples by vacuum concentration.

Table 6: Diclofenac analysis, comparison of vacuum concentrated samples

Sample	NSAID *in vitro* assay dicEQ (µg/L)	LC-MS/MS µg/L
S_{before}	0.05	0.04
S_{after}	0.15	0.15
$S_{effluent}$	1.01	0.88

4 Key Statements

- Environmental water samples should not be stored acidified in aluminum bottles as diclofenac and other NSAIDs are not stable under this condition.
- Arachidonic acid is a critical compound being sparingly soluble in aqueous buffer and sensitive to oxidation. The use of arachidonic acid in suspension with the solubilizing agent DMSO provided sufficient stability and bioavailability in the NSAID *in vitro* assay.
- The recovery of spiked diclofenac for three concentrations varied between 95 and 105 %. The accuracy of the NSAID *in vitro* assay was considered sufficient with a relative standard deviation of 2 – 8 %.
- High amount of diclofenac in surface water may result in a loss of diclofenac due to overloading the SPE column. We recommend vacuum concentration of water samples as alternative sample preparation.
- Both the chemical analytical and the effect-based *in vitro* assay method fulfilled the required maximum acceptable method detection limit for the watch list monitoring. Both methods showed similar sensitivity.
- Diclofenac is primarily responsible for the measured NSAID activity in the tested surface waters.
- 36% (LC-MS/MS) and 41% (NSAID *in vitro* assay) of the environmental samples exceeded the environmental quality standard for diclofenac.

5 List of references

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A Appendix

A.1 Analyses of sample recovery after storage in aluminum bottles at pH 3 in the freezer and at 4°C.

NSAID	cooled at 4 °C	frozen
diclofenac	69 %	92 %
indometacin	15 %	50 %
ketoprofen	92 %	92 %
ibuprofen	35 %	40 %
naproxen	38 %	76 %
salicylic acid	94 %	86 %

A.2 LC-MS/MS analysis of environmental water samples. Indicated are µg/L.

Sample number	1	2	3	4	5	6	7	8	9	10
10,11-Dihydro-10,11-dihydroxycarbamazepin	0,056	0.03	0.051	0.029	0.023	0.39	< LoQ	0.034	< LoQ	< LoQ
Bezafibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Carbamazepin	0.034	0.02	0.022	0.022	< LoQ	0.083	< LoQ	0.013	< LoQ	< LoQ
Clofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diazepam	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diclofenac	0.011	0.015	0.058	0.041	< LoQ	0.091	< LoQ	< LoQ	< LoQ	< LoQ
Etofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrinsäure	< LoQ	< LoQ	0.02	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Gabapentin	0.15	0.092	0.19	0.1	0.098	1.1	0.067	0.09	< LoQ	< LoQ
Gemfibrozil	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.029	< LoQ	0.019	< LoQ	< LoQ
Ibuprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.014	< LoQ	< LoQ	< LoQ	< LoQ
Indomethacin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Ketoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Naproxen	0.011	< LoQ	0.027	< LoQ	< LoQ	0.042	< LoQ	< LoQ	< LoQ	< LoQ

Sample number	1	2	3	4	5	6	7	8	9	10
Paracetamol	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Pentoxifyllin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Phenacetin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Primidon	0.037	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Salicylsäure	0.032	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ

Sample number	11	12	13	14	15	16	17	18	19	20
10,11-Dihydro-10,11-dihydroxycarbamazepin	0.061	0.019	0.059	0.011	< LoQ	< LoQ	0.075	0.41	0.023	0.046
Bezafibrat	< LoQ	< LoQ	0.028	< LoQ	< LoQ	< LoQ	< LoQ	0.048	< LoQ	< LoQ
Carbamazepin	0.026	< LoQ	0.078	< LoQ	< LoQ	< LoQ	0.045	0.24	0.014	0.024
Clofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diazepam	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diclofenac	0.026	0.043	0.15	0.016	< LoQ	< LoQ	0.097	0.8	0.012	0.024
Etofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Gabapentin	0.05	0.064	0.17	0.06	< LoQ	< LoQ	0.4	0.69	0.039	<0.18
Gemfibrozil	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Ibuprofen	< LoQ	0.024	0.096	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Indomethacin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Ketoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Naproxen	0.02	0.016	0.016	< LoQ	< LoQ	< LoQ	0.04	0.02	< LoQ	0.021
Paracetamol	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Pentoxifyllin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Phenacetin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Primidon	< LoQ	< LoQ	0.014	< LoQ	< LoQ	< LoQ	0.014	0.094	< LoQ	< LoQ
Salicylsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ

Sample number	21	22	23	24	25	26	27[S]	28	29	30
10,11-Dihydro-10,11-dihydroxycarbamazepin	0.084	0.062	0.033	0.026	0.023	< LoQ	< LoQ	0.035	< LoQ	0.016
Bezafibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Carbamazepin	0.038	0.03	0.015	0.011	0.013	< LoQ	< LoQ	0.015	< LoQ	0.02
Clofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diazepam	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diclofenac	0.03	< LoQ	0.011	0.011	0.019	< LoQ	< LoQ	0.12	< LoQ	0.026
Etofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Gabapentin	0.24	0.18	0.065	0.047	0.097	0.047	< LoQ	0.14	< LoQ	0.045
Gemfibrozil	0.012	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Ibuprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Indomethacin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Ketoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Naproxen	0.029	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.026	< LoQ	0.011
Paracetamol	< LoQ	< LoQ	0.013	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Pentoxifyllin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Phenacetin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Primidon	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Salicylsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ

[S] internal standards were totally suppressed

Sample number	31	32	33	34	35	36	37	38	39	40
10,11-Dihydro-10,11-dihydroxycarbamazepin	0.016	0.09	0.16	0.081	0.15	0.017	< LoQ	0.079	0.1	0.16
Bezafibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Carbamazepin	< LoQ	0.033	0.044	0.036	0.069	< LoQ	< LoQ	0.013	0.02	0.064

Sample number	31	32	33	34	35	36	37	38	39	40
Clofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diazepam	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Diclofenac	< LoQ	0.063	0.17	0.082	0.31	< LoQ	< LoQ	0.023	< LoQ	0.077
Etofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrat	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.017
Fenoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Gabapentin	0.047	0.16	0.26	0.14	0.34	0.048	< LoQ	0.24	0.3	0.33
Gemfibrozil	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.017
Ibuprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Indomethacin	< LoQ	< LoQ	0.014	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Ketoprofen	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Naproxen	< LoQ	0.038	0.1	0.03	0.067	< LoQ	< LoQ	< LoQ	< LoQ	0.038
Paracetamol	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Pentoxifyllin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.12
Phenacetin	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ
Primidon	< LoQ	< LoQ	0.015	< LoQ	0.019	< LoQ	< LoQ	< LoQ	< LoQ	0.028
Salicylsäure	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	0.075	< LoQ

Sample number	41	42	43	44
10,11-Dihydro-10,11-dihydroxycarbamazepin	0.07	0.1	0.15	0.34
Bezafibrat	< LoQ	< LoQ	< LoQ	< LoQ
Carbamazepin	0.027	0.046	0.075	0.16
Clofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ
Diazepam	< LoQ	< LoQ	< LoQ	< LoQ
Diclofenac	0.011	0.024	0.018	0.17
Etofibrat	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrat	< LoQ	< LoQ	< LoQ	< LoQ
Fenofibrinsäure	< LoQ	< LoQ	< LoQ	< LoQ

Sample number	41	42	43	44
Fenoprofen	< LoQ	< LoQ	< LoQ	< LoQ
Gabapentin	0.24	0.3	0.46	0.85
Gemfibrozil	< LoQ	< LoQ	< LoQ	< LoQ
Ibuprofen	< LoQ	< LoQ	< LoQ	< LoQ
Indomethacin	< LoQ	< LoQ	< LoQ	0.011
Ketoprofen	< LoQ	< LoQ	< LoQ	0.024
Naproxen	< LoQ	0.013	< LoQ	0.038
Paracetamol	< LoQ	< LoQ	< LoQ	< LoQ
Pentoxifyllin	< LoQ	< LoQ	< LoQ	< LoQ
Phenacetin	< LoQ	< LoQ	< LoQ	< LoQ
Primidon	0.011	0.023	0.042	0.051
Salicylsäure	< LoQ	< LoQ	< LoQ	< LoQ

A.3 Analysis of NSAID activity by the NSAID in vitro assay of environmental water samples. Indicated are dicEQ (µg/L diclofenac).

sample number	1	2	3	4	5	6	7	8	9	10
dicEQ	0.021	0.025	0.076	0.047	0.037	0.098	< LoD	0.032	< LoD	< LoD

sample number	11	12	13	14	15	16	17	18	19	20
dicEQ	0.032	0.044	0.159	0.032	< LoD	< LoD	0.103	0.485	0.014	0.012

sample number	21	22	23	24	25	26	27	28	29	30
dicEQ	0.026	< LoD	0.015	0.018	0.024	0.019	0.016	0.087	0.016	0.024

sample number	31	32	33	34	35	36	37	38	39	40
dicEQ	0.013	0.057	0.147	0.056	0.135	0.012	< LoD	0.043	0.018	0.065

sample number	41	42	43	44
dicEQ	0.031	0.044	0.037	0.223