Spatial and temporal variations in anti-androgenic activity and environmental risk in a small river

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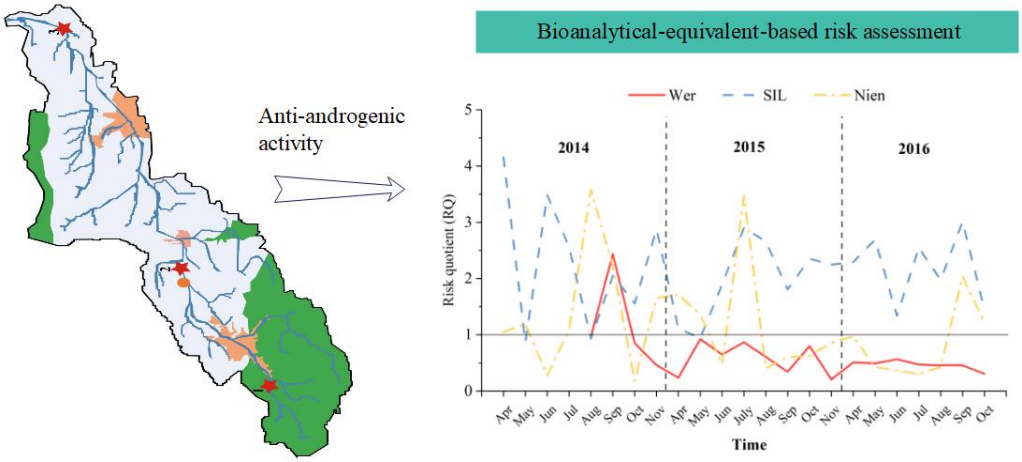
Anti-androgenic activity
Bioanalytical-equivalent-based risk assessment
Wer
SIL
Nien
2014
2015
2016
Risk quotient (RQ)
Time

20

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- Spatial and temporal variations in anti-androgenic activity were observed.
- A bioanalytical-equivalent-based risk assessment method was developed.
- The WWTP continuously discharged anti-androgens into the river.
- High environmental risk was observed at the site directly influenced by effluents.
- The water quality showed an improved trend in terms of anti-AR activity.

Abstract

The biological effects of multiple compounds have been widely investigated in aquatic environments. However, investigations of spatial and temporal variations in biological effects are rarely performed because they are time-consuming and labor-intensive. In this study, the variability of the anti-androgen, receptor-mediated activity of surface water samples was observed over 3 years using *in vitro* bioassays. Large-volume water samples were collected at one site upstream (Wer site) and two sites downstream (Sil and Nien sites) of a wastewater treatment plant (WWTP) outfall in the Holtemme River. Anti-AR activity was persistently present in all surface water samples over the three years. Large spatial variations in anti-androgenic activity were observed, with the lowest activity at the Wer site (mean concentration of 9.5 ±7.2 μg flutamide equivalents per liter) and the highest activity at the Sil site (mean concentration of 31.1 ± 12.0 μg flutamide equivalents per liter) directly influenced by WWTP effluents. On the temporal scale, no distinct trend for anti-AR activity was observed among the seasons in all three years. The anti-androgenic activity at the upstream Wer site showed a decreasing trend from 2014 to 2016, indicating improved water quality. A novel bioanalytical-equivalent-based risk assessment method considering the frequency of risk occurrence was developed and then utilized to assess the environmental risk of anti-androgenic activity in the Holtemme River. The results revealed that the highest risk was present at the Sil site, while the risk was considerably reduced at the Nien site. The risk at the upstream Wer site was the lowest.

Keywords: Cell viability; Antiandrogen; *In vitro* bioassay; Risk assessment

1. Introduction

Different classes of compounds are considered anti-androgens, which can disturb the development of the male phenotype and secondary sexual characteristics in humans and other vertebrates, including the pharmaceuticals flutamide and 17α-ethinyl-estradiol, the plastic component bisphenol A, the biocide triclosan, the pesticides linuron and vinclozolin, the fluorescent dye 4-methyl-7-diethylaminocoumarin, and the polycyclic aromatic hydrocarbon (PAH) metabolite 1-hydroxypyrene (Di Paolo et al., 2016a; Muschket et al., 2018; van Vugt-Lussenburg et al., 2018). These contaminants in aquatic ecosystems originate from numerous domestic, industrial, and farming applications and ultimately enter water bodies through discharges of effluents from wastewater treatment plants (WWTPs) due to their limited effectiveness in removing contaminants (Katsiadaki et al., 2012; Di Marcantonio et al., 2020; Houtman et al., 2021). Therefore, anti-androgens in aquatic ecosystems have become concerning (Muschket et al., 2018; Sauer et al., 2018).

In vitro and *in vivo* bioassays are useful tools for analyzing the toxicological burden of complex chemical mixtures (Kassotis et al., 2015; Di Paolo et al., 2016b; Heinrich et al., 2017; Zhou et al., 2021). Among the *in vitro* assays for the detection of (anti-)androgenic or (anti-)estrogenic activity, chemically activated luciferase gene expression (CALUX) assays can detect the activation of androgen or estrogen receptors at low concentrations (Weiss et al., 2009; van der Burg et al., 2010; Brand et al., 2013; Alvarez-muñoz et al., 2015; Välitalo et al., 2016). These bioassays are well developed and have been widely utilized in previous studies (Brand et al., 2013; Escher et al., 2014;

van Vugt-Lussenburg et al., 2018).

The bioassay-derived activity of a sample can be expressed using the bioanalytical equivalent (BEQ), which is the concentration of a known reference standard that elicits the same response as the tested sample (Zhou et al., 2021). Antiandrogen receptor-mediated (anti-AR) activity induced by anti-androgens has been detected in effluents (Van Der Linden et al., 2008; Jálová et al., 2013), oceans (Alvarez-muñoz et al., 2015), and rivers (Urbatzka et al., 2007; Van Der Linden et al., 2008; Weiss et al., 2009; Zhao et al., 2011; Liscio et al., 2014; Kassotis et al., 2015, 2016; Muschket et al., 2018; Houtman et al., 2021). However, most studies regarding anti-AR activity in aquatic systems have mainly concentrated on short-term surveys (Zhao et al., 2011) or have only been conducted at a spatial resolution (Urbatzka et al., 2007; Kassotis et al., 2015, 2016). The spatial and temporal variations in anti-androgenic activity have been sparsely investigated.

For risk assessment of a single compound, a detected concentration exceeding the predicted no effect concentration (PNEC) indicates a potential environmental risk (Desbiolles et al., 2018; Zhou et al., 2019). Similar to this regulative approach, the hazardous potential or risk of an environmental sample usually containing mixtures of several compounds with putative environmental impacts can be calculated by dividing the bioanalytical equivalent (BEQ) of the sample by the environmental quality standard (EQS) (Kase et al., 2018) for each receptor or endpoint (in the case of *in vitro* assays) or for each species group, such as fish, daphnia, or algae (in the case of *in vivo* assays). This approach characterizes the risk of samples under the conditions of bioassay-

derived effects but disregards the frequency of risk occurrence. Notably, certain biological effects that are long-term and widely present in aquatic ecosystems have a deeper impact than those that are short-term or small-scale (Zhou et al., 2019). Therefore, it is necessary to consider the frequency of risk occurrence in hotspot identification or priority pollutant screening (Tousova et al., 2017; Desbiolles et al., 2018; Zhou et al., 2019).

The Holtemme River (Saxony Anhalt, Germany) was identified as a hotspot of anti-androgenic effects, and 4-methyl-7-diethylaminocoumarin (C47) and two derivatives have been confirmed as the drivers of this effect (Muschket et al., 2018). Recently, the longitudinal distribution and fate of these novel anti-androgens in the impacted river have been investigated (Muschket et al., 2021). The purposes of this study were (1) to investigate the spatial and temporal variations in anti-AR activity in aquatic ecosystems using the Holtemme River as a case study after three years of sampling from 2014 to 2016 and (2) to develop a reliable method that could quantify the potential risk of biological responses, especially on a wide spatial and temporal scale.

2. Materials and methods

2.1 Materials

The chemicals dihydrotestosterone (DHT), 3–[4,5–dimethylthiazol–2–yl]–2,5–diphenyltetrazolium bromide (MTT), and flutamide with a purity of ⩾97%, the solutions dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS), and the assay medium Dulbecco's Modified Eagle's Medium and Ham's F12 medium (DMEM/F12) without phenol red, as well as the supplementary substances fetal calf

serum (FCS), penicillin/streptomycin solution, nonessential amino acids and G418 antibiotic were purchased from Sigma–Aldrich Chemie GmbH (Darmstadt, Germany). Stock solutions were prepared with DMSO and stored at –20 °C.

2.2 Sample collection

The Holtemme River (Saxony-Anhalt, Germany) originates from Harz National Park and flows into the Bode River (Weitere et al., 2021). Surface water samples were obtained from 2014 to 2016 at the Wernigerode (Wer), Silstedt (Sil) and Nienhagen (Nien) sites in the river (Fig. 1). The first site, Wer, was located at Steinerne Renne upstream of the town of Wernigerode with minimum human disturbance; thus, samples from this site were selected as references. The second site, Sil, was located approximately 1.3 km downstream of the effluent of the WWTP in Silstedt. The third site, Nien, was located approximately 25 km downstream of the WWTP. Twenty to fifty liters of water samples were extracted on-site using a large volume solid phase extraction (LVSPE) device according to the previously reported methods (Schulze et al., 2017; Välitalo et al., 2017). A total of 65 water samples were collected at the three sites in spring, summer, and autumn. Sampling was not performed in winter due to low temperatures. The concentration of the sample was expressed as the relative enrichment factor (REF), which is the ratio of the volume of a water sample to the final volume in bioassays (Escher et al., 2021). Four blank controls and six solvent controls were also prepared and stored in a -20 °C refrigerator.

2.3 Cytotoxicity and CALUX assays

MTT assays were performed using the human osteosarcoma U2OS cell line obtained

from BioDetection Systems BV (BDS, Amsterdam, The Netherlands) to observe the cytotoxicity of water sample extracts (Otto et al., 2008). Briefly, cells in an assay medium (DMEM/F12 supplemented with 5% stripped FCS, 0.2% penicillin/streptomycin solution and 1% nonessential amino acids) were seeded into 96-well plates at a density of 10,000 cells/well (Di Paolo et al., 2016a). After 24 h of incubation (37 °C and 5% CO_2), the medium was replaced with the exposure medium containing the tested samples, and the concentration of DMSO was 1% (v/v). After 24 h of exposure, the medium was removed, and the cells were washed in 150 µL/well of PBS and then incubated with the MTT solution for 30 min. Afterward, the solution was discarded, and 200 µL/well DMSO was added prior to shaking for 20 min. The absorbance (492 nm) in the microplates was measured using a multiwell plate reader (Tecan, Crailsheim, Germany).

CALUX assays were performed in the range of concentrations without cytotoxicity (≥90% cell viability) according to the BioDetection Systems protocol with slight modifications (Di Paolo et al., 2016a). Seeded U2OS cells were incubated for 24 h in the exposure medium, and then the medium was removed and cells were lysed in 30 µL/well of Triton-lysis buffer. The amount of luciferase activity was quantified using a luminometer (GloMax®96 Microplate Luminometer, Germany). For the anti-AR CALUX assay, the same procedure applied to the AR CALUX assay was followed, with the exception that the exposure medium was supplemented with the agonist DHT at a concentration of 4.2×10^{-10} mol/L. At least three independent technical replicates were tested for every LVSPE sample.

2.4 Risk assessment

In general, the potential risk of anti-AR activity in surface waters was assessed according to Equation 1. A risk quotient (RQ) equal to or greater than one expresses a high environmental risk. BEQ is the flutamide equivalent (flu-eq.) of a water sample to inhibit androgenic activity as the reference flutamide at the specific effect level IC_{50} (Equation 2, Zhou et al., 2021). As recommended by Escher et al. (2018), the EQS of anti-AR activity in surface waters (14.4 μg flu-eq./L) for ecology and human health was selected for environmental risk assessment.

$$RQ = \frac{BEQ}{EQS} \quad (1)$$

$$BEQ = \frac{EC_{50,flu}}{EC_{50,sample}} \quad (2)$$

where $EC_{50,flu}$ is the flutamide concentration that causes 50% receptor inhibition between the negative controls (0% receptor inhibition) and the maximum inhibition caused by the reference flutamide (100% receptor inhibition) and $EC_{50,sample}$ is the sample concentration that causes 50% receptor inhibition between the negative controls and the maximum inhibition caused by the sample.

For bioassay results with large temporal and spatial variations, the risk assessment should consider the frequency of risk occurrence. Therefore, an optimized risk quotient (RQ_f) initially developed for priority chemical screening (Equations 3 and 4)(Zhou et al., 2019) was extended for risk assessment based on bioassay results. Here, RQ_f denoted the ratio of the mean BEQ (BEQ_{mean}) to the EQS after considering the frequency (F) of BEQs exceeding the EQS. RQ_f was classified into 5 groups: $RQ_f \geq 1$ means high environmental risk; $1 > RQ_f \geq 0.1$ means moderate environmental risk; $0.1 >$

184 $RQ_f \geq 0.01$ means small-scale or occasional adverse effect; $0.01 > RQ_f > 0$ means a
185 quite limited effect; and $RQ_f = 0$ means no negative effects, according to our previous
186 publication (Zhou et al., 2019).

187 $$RQ_f = \frac{BEQ_{mean}}{EQS} \times F \quad (3)$$

188 $$F = \frac{NO1}{NO2} \quad (4)$$

189 where NO1 represents the number of samples with BEQ values higher than the EQS
190 and NO2 represents the total number of samples.

191 2.5 Statistical analysis

192 For the calculation of the IC_{50}, dose–response curves for anti-AR CALUX were
193 computed by four-parameter logistic regressions with variable slopes (Prism 6.0,
194 GraphPad Software Inc., San Diego, USA).

195 T tests or one-way ANOVAs followed by a post hoc test (Fisher's least-square
196 difference test, two-tailed) were performed using SPSS Statistics 17.0 (SPSS Inc., USA)
197 to identify significant differences among samples. All figures were plotted using Origin
198 2021 (OriginLab, Hampton, USA).

199 **3. Results and discussion**

200 3.1 Cytotoxicity

201 In the case of antagonistic activity, cell viability was measured with MTT assays to
202 determine whether the inhibition of responses in the anti-AR CALUX was attributed to
203 receptor-mediated antagonisms instead of nonspecific cytotoxicity (Houtman et al.,
204 2021). For Wer samples, a significant decrease in cell viability was observed at a REF
205 of 100 in 2014 (Fig. 2), and no significant decrease was observed in the next two years.

For samples collected at the Sil site, higher cytotoxicity was observed in 2014 than in 2016. However, at the Nien site, higher toxicity was observed in 2016 than in 2014. Generally, 17 of 24 (71%) Sil samples, 10 of 23 (43%) Nien samples, and 7 of 18 (39%) Wer samples showed cell viability <80% at a REF of 100 over all three years. Among all three sites, the highest cytotoxicity was observed at the Sil site, approximately 1.3 km downstream of the WWTP. The cytotoxicity at the Nien site (25 km downstream of the WWTP) was similar to that at the Wer site upstream of the WWTP. For the anti-AR CALUX assays, the maximum REF was set without cytotoxicity (cell viability ≥ 90%). No cytotoxicity was observed for the blanks and solvent controls.

3.2 Antiandrogen receptor-mediated (anti-AR) activity

Spatial and temporal variations in anti-AR activity were observed at three sites in the Holtemme River from April to November in 2014, 2015 and 2016 (Fig. 3). For most of the samples at the same sampling time, the lowest activity was observed at the upstream Wer site, with a mean value of 9.5 ± 7.2 μg/L flu-eq. in all three years. The highest anti-AR activity mainly appeared at the WWTP downstream of the Sil site, with a mean value of 31.1 ± 12.0 μg/L flu-eq., presumably due to long-lasting wastewater discharge. Anti-AR activity decreased farther downstream of the Nien site (18.4 ± 13.1 μg/L flu-eq.), probably due to in-stream degradation and dilution. With the exception of one sample in September 2014, almost all samples showed significant differences in anti-AR activity between the Wer site and the Sil site. More than half of the samples showed significant differences in anti-AR activity between the Wer site and the Nien site. Blanks and solvent controls were negative for the anti-AR CALUX assays.

In 2014, no significant difference in anti-AR activity was observed between the upstream Wer site (17.0 ± 10.8 μg/L flu-eq.) and the downstream Sil (32.9 ± 15.5 μg/L flu-eq.) and Nien (20.8 ± 15.2 μg/L flu-eq.) sites. However, a significant difference was observed between the Wer site (8.0 ± 3.9 μg/L flu-eq.) and the Sil site (29.0 ± 9.5 μg/L flu-eq.) in 2015 ($p<0.05$), but this difference was not obvious between the Wer site and the Nien site (16.9 ± 14.0 μg/L flu-eq.). In 2016, a significant increase ($p<0.01$) in anti-AR activity was observed at the downstream Sil (32.5 ± 8.3 μg/L flu-eq.) and Nien (17.6 ± 5.1 μg/L flu-eq.) sites compared with the activity at the upstream Wer site (6.7 ± 1.1 μg/L flu-eq.). Although no obvious difference in anti-AR activity was observed at two downstream sites among the different years, the anti-AR activity at the upstream Wer site showed a decreasing trend during the study period. These variations might be due to a steady discharge of antiandrogens into the river from the WWTP at the Sil site, but a decrease in the input of antiandrogens through nonpoint source pollution at the upstream Wer site. Anti-AR activity continuously occurred in all surface water samples and fluctuated among the seasons in all three years, but no distinct trend was observed, indicating that anti-androgens have a widespread and stable presence in the Holtemme River.

Commonly, there is a gap between bioassay-derived BEQs and chemically analyzed BEQs, which is considered caused by unidentified chemicals and other factors (Zhou et al., 2021), and unidentified chemicals may have a key role (Escher et al., 2020; Zhou et al., 2021). Eight compounds, namely, tebuconazole, bicalutamide, diuron, bisphenol S, tonalide, genistein, daidzein, and 2,4-dinitrophenol, are known inhibitors of

androgenic activity (Escher et al., 2017; Muschket et al., 2021). The chemical analysis results for the 2014 water samples (unpublished data) showed that the concentrations of tebuconazole, diuron, genistein, bisphenol S, tonalide, and daidzein were less than 32 ng/L, that the concentrations of bicalutamide and 2,4-dinitrophenol were below 103 ng/L, and that none of these chemicals was detected at the Wer site, suggesting that WWTP effluents were responsible for the increased concentrations. Peak concentrations observed at the Nien site were lower than those at the Sil site, indicating a possible dilution downstream of the WWTP (Beckers et al., 2020). These low-concentration chemicals might not explain the detected anti-AR activity, which supports the hypothesis that nonanalyzed or unknown (natural or anthropogenic) compounds can significantly contribute to the toxicity.

Muschket and coworkers performed an effect-directed analysis (EDA) of Holtemme River water and identified that C47 and its transformation products 4-methyl-7-ethylaminocoumarin (C47T1) and 4-methyl-7-aminocoumarin (C47T2) discharged from the WWTP of Silstedt are novel antiandrogens that continuously occurred in surface waters at the Sil and Nien sites from 2014 to 2016 (Muschket et al., 2021, 2018) but not at the Wer site. The anti-androgenic potency of C47 and C47T1 was greater than that of the reference flutamide (Muschket et al., 2018). At the Sil site, the mean concentrations of C47 (8.4 µg/L flu-eq) and C47T1 (2.6 µg/L flu-eq) (Muschket et al., 2021) contributed 27% and 8%, respectively, of the anti-androgenic activity. The anti-androgenic potency of C47T2 was 5 times lower than that of the reference flutamide (Muschket et al., 2018). The concentration of C47T2 was equal to 0.1 µg/L flu-eq. with

a minimal contribution to the activity. Therefore, C47 and C47T1 discharged from the WWTP of Silstedt were the major drivers of the increased anti-AR activity at the Sil site. The concentrations of C47 and transformation products greatly decreased at the Nien site (Muschket et al., 2018), and thus, anti-AR activity also decreased. The spatial and temporal variations in anti-AR activity in the Holtemme River might be attributed to a) the release of anti-androgens such as C47 and transformation products from WWTPs (Muschket et al., 2018), b) the dilution and degradation of anti-androgens after discharge into the river (Houtman et al., 2021; Muschket et al., 2021), and c) diffuse and random inputs via small point sources and groundwater input (Burns et al., 2018; Beckers et al., 2020).

The levels of anti-androgenic activity in the Holtemme River were comparable to those reported for surface waters in the Netherlands, where anti-AR activity varied in the range of 0 to 90 μg/L flu-eq. at three sites that served as abstraction sites for drinking water production (Houtman et al., 2021). Comparable anti-AR activity (2 to 48 μg/L flu-eq.) was detected in Missouri surface waters, USA (Kassotis et al., 2015). Higher anti-androgenic activity was detected in other areas. For example, anti-androgenic activity in water samples from the Lambro River in Italy ranged from 370 to 4723 μg/L flu-eq. (Urbatzka et al., 2007); anti-androgenic activity in surface water at a West Virginia injection well disposal site in the US reached 700 μg/L flu-eq. (Kassotis et al., 2016); the levels of anti-androgenic activity in the Pearl River contaminated by effluents and raw sewage in China were in the range of 20.4 to 935 μg/L flu-eq. (Zhao et al., 2011). Anti-AR activity was also detected in WWTPs (Jálová et al., 2013), and

high concentrations of C47 and its transformation products were observed in WWTP effluents (Muschket et al., 2021).

Notably, almost all the above mentioned results are obtained without considering metabolism, since the metabolic capacity of the CALUX system is quite weak and can be disregarded. However, metabolism is quite important for detoxifying and even activating certain compounds (Brack et al., 2016; Jacobs et al., 2013; Mollergues et al., 2017; van Vugt-Lussenburg et al., 2018). The anti-androgenic activity of samples after metabolism is still unknown, and further research is still needed.

3.3 Risk assessment based on bioassay results

Biological responses obtained by *in vivo*/*in vitro* bioassays using target organisms potentially have relevance to environmental risks on nontarget organisms. Risk assessment based on effect-based methods can serve as a tool to estimate the effects of compounds on environmental and human health and to identify hotspots for further analysis (Kase et al., 2018). At the Wer site, which was considered less contaminated, only 1 of 18 samples (i.e., 5.6%) had an RQ above one. However, 21 of 24 (i.e., 88%) Sil samples and 11 of 23 (i.e., 48%) Nien samples had RQ values equal to or greater than one (Fig. 4). Notably, although potential environmental risk was observed at the three sites, the frequencies of risk occurrence were greatly different. Therefore, we developed an optimized risk assessment method considering the frequencies of risk occurrence and further verified the reliability of this method in this study. The value of RQ_f at the Wer site was 0.04, implying occasional adverse effects at the upstream control site. The RQ_f value for the Sil samples was 1.8, indicating a high environmental

risk at the downstream discharge site. The RQ_f value for the Nien sample was 0.6, indicating a moderate environmental risk. These results suggest that the WWTP affected the receiving Holtemme River, and after 25 km of transport and dilution, the potential risk of anti-AR activity greatly decreased at the Nien site.

Although the RQ_f values at the Sil site did not undergo a substantial change from 2014 to 2016, the RQ_f values at the Wer and Nien sites showed a decreasing trend during the sampling period (Table 1). In general, the water quality of the Holtemme River showed an improving trend in terms of anti-AR activity, but WWTPs at the Sil site stably discharged anti-androgens into the river. The potential adverse effects of anti-androgens on aquatic organisms have received attention in previous studies (Jensen et al., 2004; Urbatzka et al., 2007; Katsiadaki et al., 2012). For example, exposure to 651 μg/L flu-eq. caused a significant decrease in the fecundity of fathead minnows. In addition, embryo hatching was reduced at this concentration (Jensen et al., 2004); a field study showed the presence of anti-androgens in three-spined sticklebacks in a river receiving WWTP effluents in the UK (Katsiadaki et al., 2012). Thus, the anti-androgenic load of the Holtemme River after receiving effluent may also pose a risk to aquatic environments.

4. Conclusions

Spatial and temporal variations in cytotoxicity and anti-androgenic activity were observed over a 3-year period from upstream to downstream of the Holtemme River. This study emphasizes that the effects of wastewater discharge on cytotoxicity and anti-androgenic activity are evident over a certain distance. The highest cytotoxicity and

anti-androgenic activity were detected downstream of the effluent, and after approximately 25 km of dilution and degradation, cytotoxicity and anti-androgenic activity greatly decreased. From 2014 to 2016, the cytotoxicity and anti-androgenic activity at the upstream Wer site showed a decreasing trend, indicating improved water quality. However, the effects of WWTP effluents were still evident. In this study, an optimized risk assessment method originally designed for chemical analysis data was extended for risk assessment of bioassay data. The results showed that the RQ_f at the Sil site near the WWTP effluent was much higher than those at the upstream Wer site and downstream Nien site far from WWTP disturbance.

CRediT authorship contribution statement

Shangbo Zhou: Writing - Original draft, Formal analysis, Visualization. Tobias Schulze: Conceptualization, Investigation, Writing - review & editing. Werner Brack: Conceptualization, Investigation, Writing - review & editing. Thomas-Benjamin Seiler: Conceptualization, Formal analysis, Writing - review & editing. Henner Hollert: Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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543 Table 1 Temporal variations in RQ_f values at three sites from 2014 to 2016

Sites	Year	NO1	NO2	F(%)	RQ	RQ_f
Wer						
	2014	1	4	25	1.20	0.30
	2015	0	7	0	0.57	0.00
	2016	0	8	0	0.43	0.00
Sil						
	2014	6	8	75	2.31	1.73
	2015	7	8	88	1.98	1.74
	2016	8	8	100	2.19	2.19
Nien	2014	6	8	75	1.40	1.05
	2015	3	8	38	1.18	0.45
	2016	2	7	29	0.89	0.25

544

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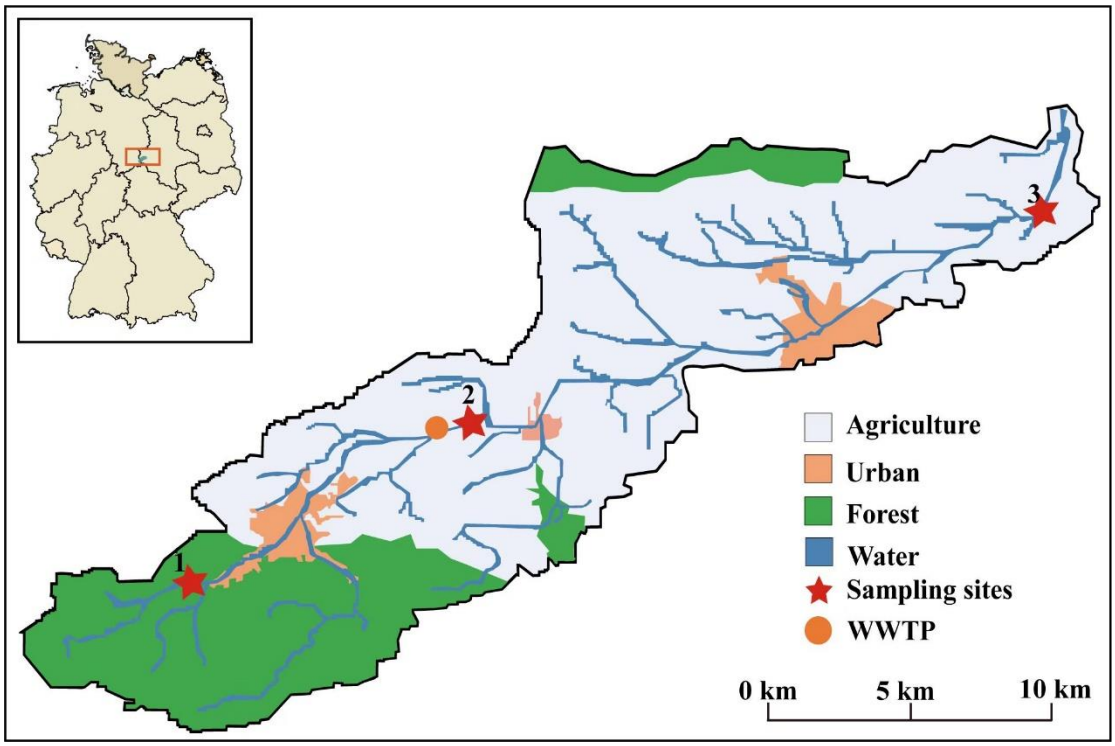

546

547 Fig. 1 Map of the catchment of the Holtemme River showing sampling sites (revised

548 from Muschket et al., 2021). Site 1: Wernigerode; Site 2: Silstedt; Site 3: Nienhagen;

549 WWTP: wastewater treatment plant.

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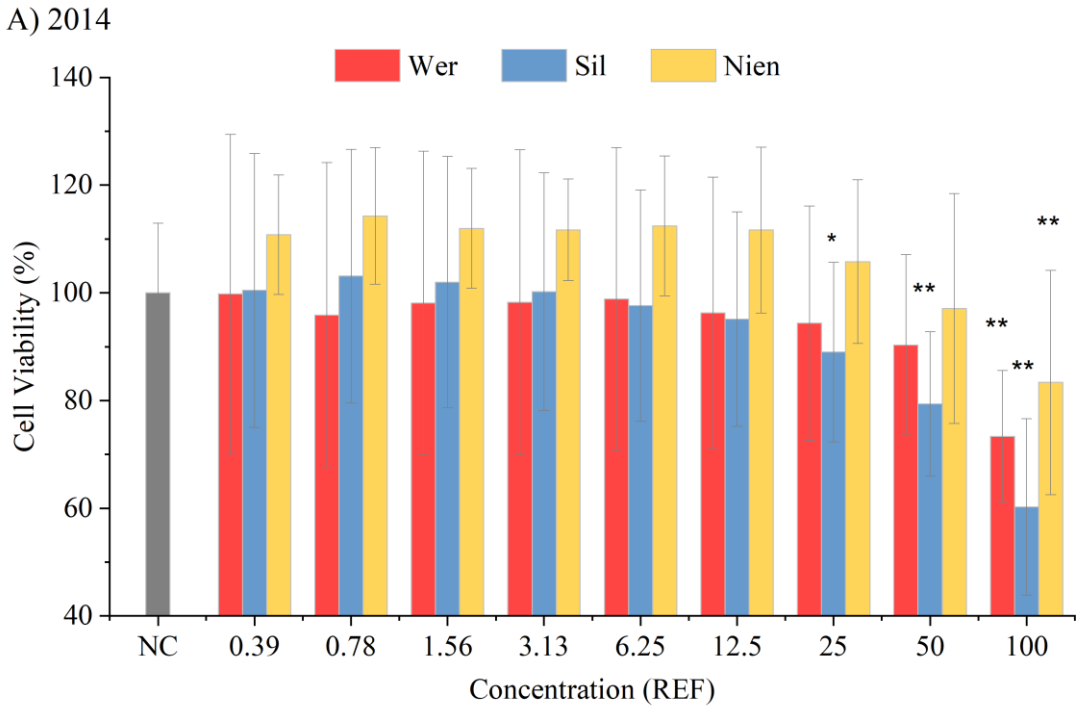
A) 2014
Wer
Sil
Nien
Cell Viability (%)
140
120
100
80
60
40
NC
0.39
0.78
1.56
3.13
6.25
12.5
25
50
100
Concentration (REF)
*
**
**
**
**

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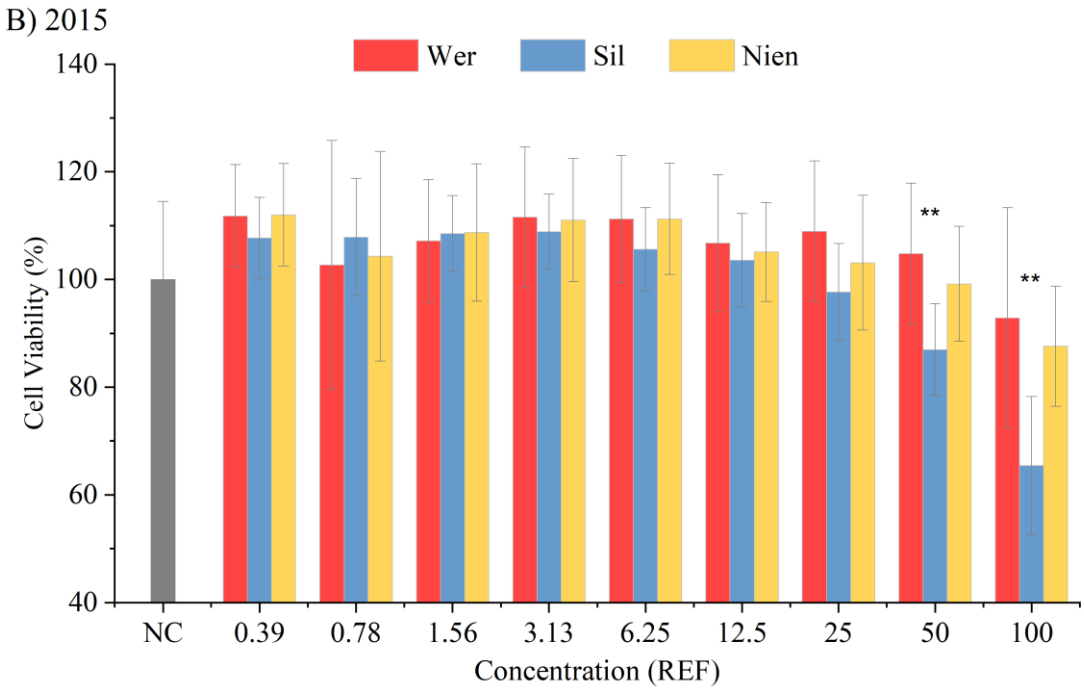
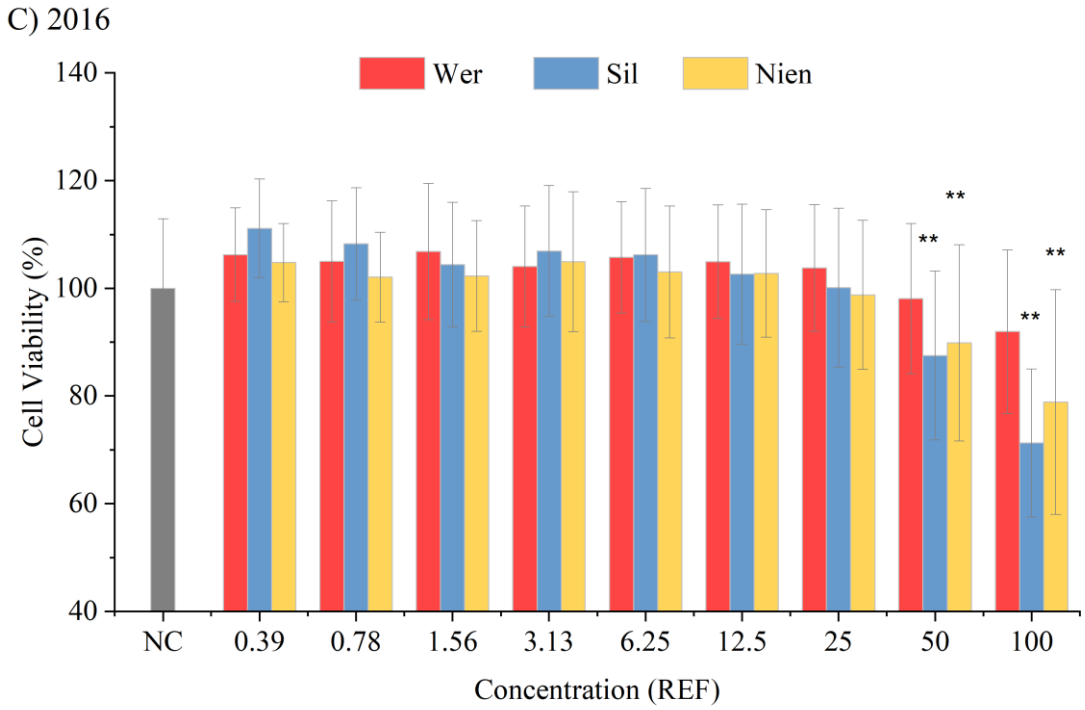

B) 2015
Wer
Sil
Nien
Cell Viability (%)
140
120
100
80
60
40
NC
0.39
0.78
1.56
3.13
6.25
12.5
25
50
100
Concentration (REF)
**
**

552



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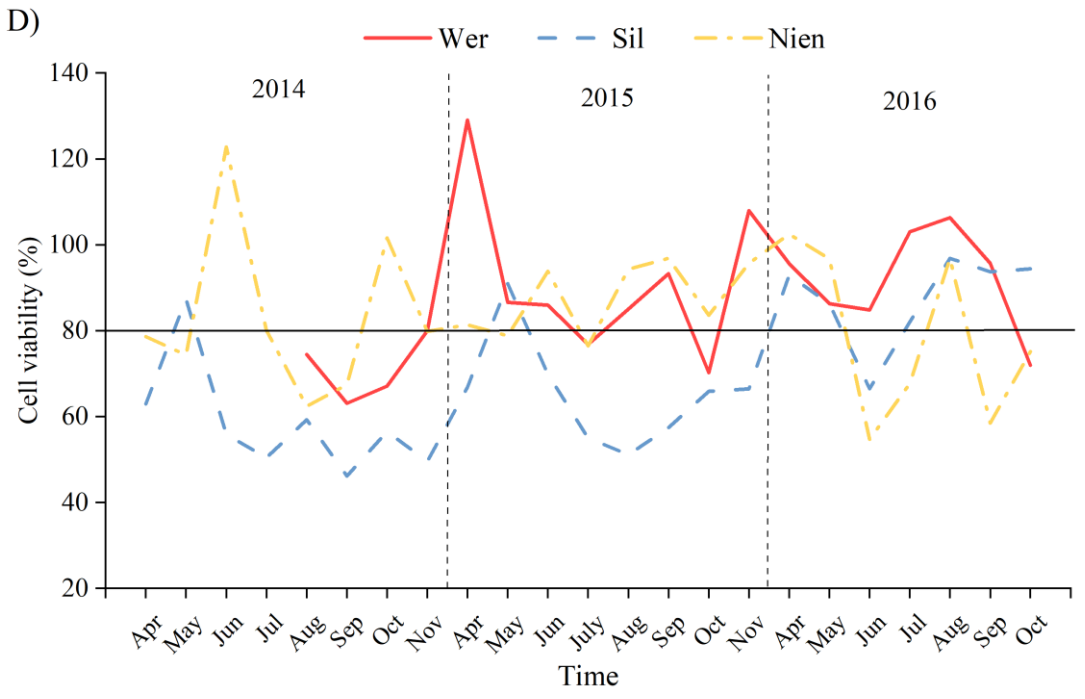
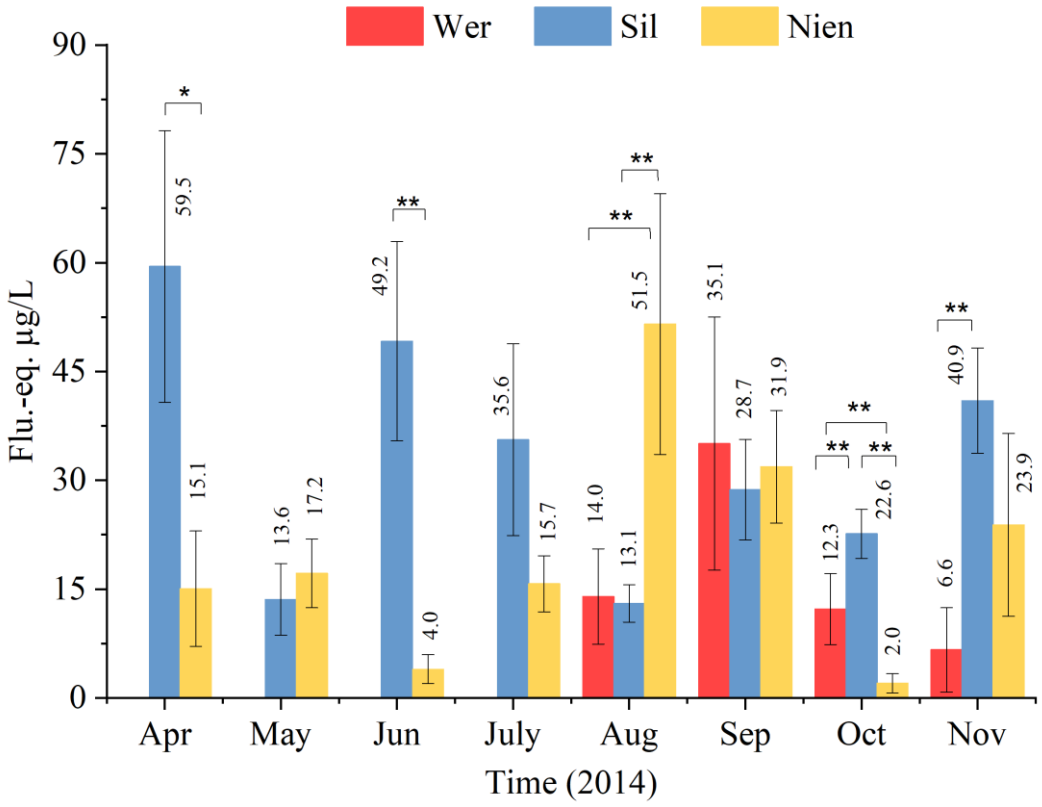


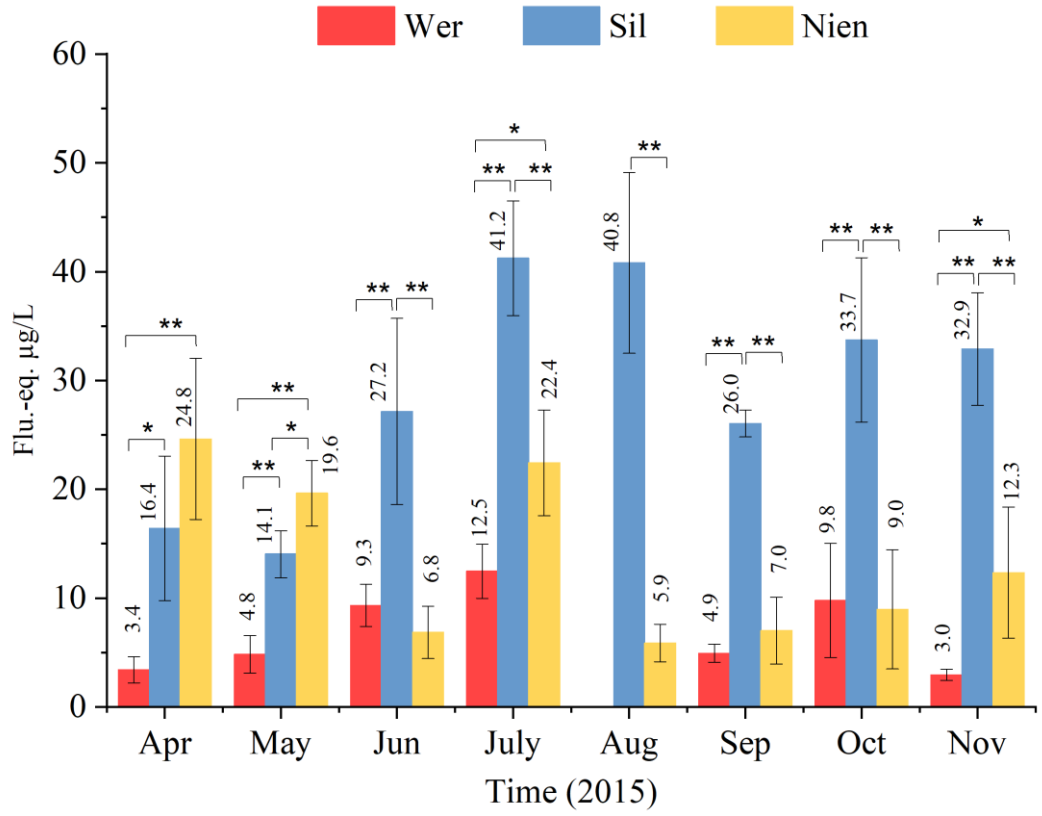
554

555 Fig. 2 Cell viability of water samples at the three sites (Wer, Sil and Nien) from 2014
556 to 2016. Responses are expressed as a % of the relative response to the negative control
557 (NC). A) means cell viability in 2014; B) means cell viability in 2015; C) means cell
558 viability in 2016; D) temporal variations in cell viability at the three sites, and cell
559 viability is given at a concentration of REF 100. Data are presented as the

mean ± standard deviation (SD). * represents a significant difference ($p<0.05$) with respect to indicators compared with the negative control. ** represents an extremely significant difference. Error bar, standard deviation ($n\geq12$); DMSO was used as the negative control.


Wer
Sil
Nien
Flu.-eq. µg/L
Time (2014)
Apr
May
Jun
July
Aug
Sep
Oct
Nov
3.4
16.4
24.8
4.8
14.1
19.6
9.3
27.2
6.8
12.5
41.2
22.4
14.0
13.1
51.5
35.1
28.7
31.9
12.3
22.6
2.0
6.6
40.9
23.9

565


Wer
Sil
Nien
Flu.-eq. µg/L
Time (2015)
Apr
May
Jun
July
Aug
Sep
Oct
Nov
3.4
16.4
24.8
4.8
14.1
19.6
9.3
27.2
6.8
12.5
41.2
22.4
40.8
5.9
4.9
26.0
7.0
9.8
33.7
9.0
3.0
32.9
12.3

566

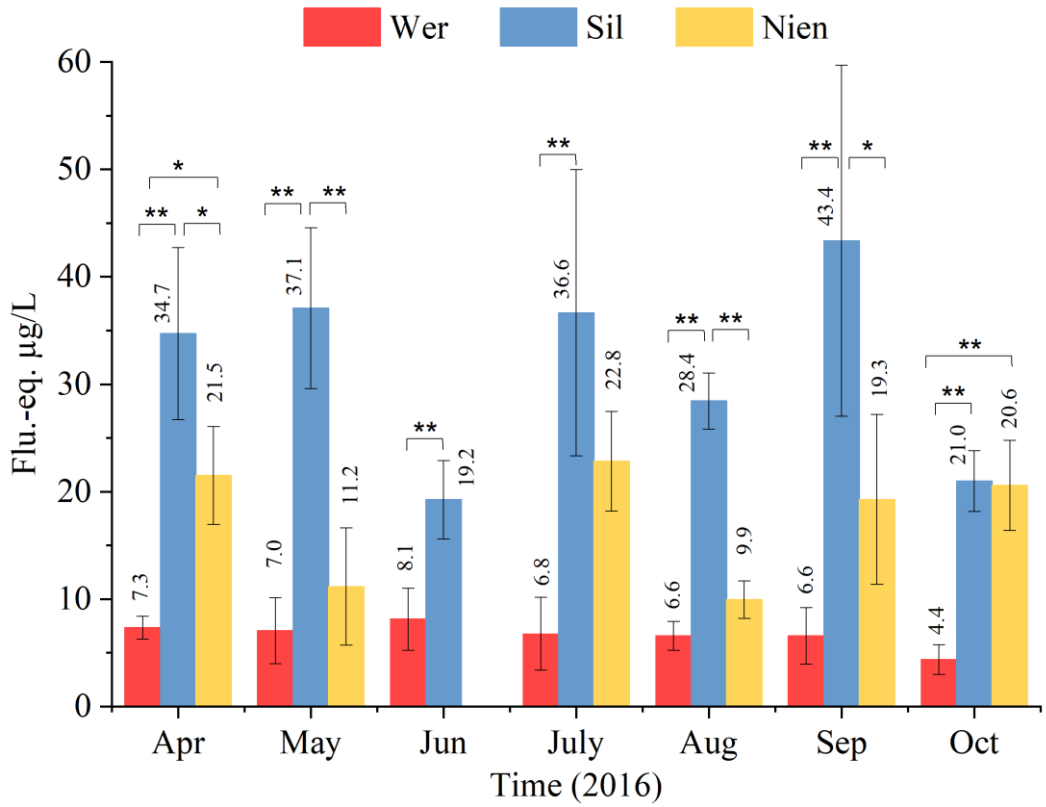
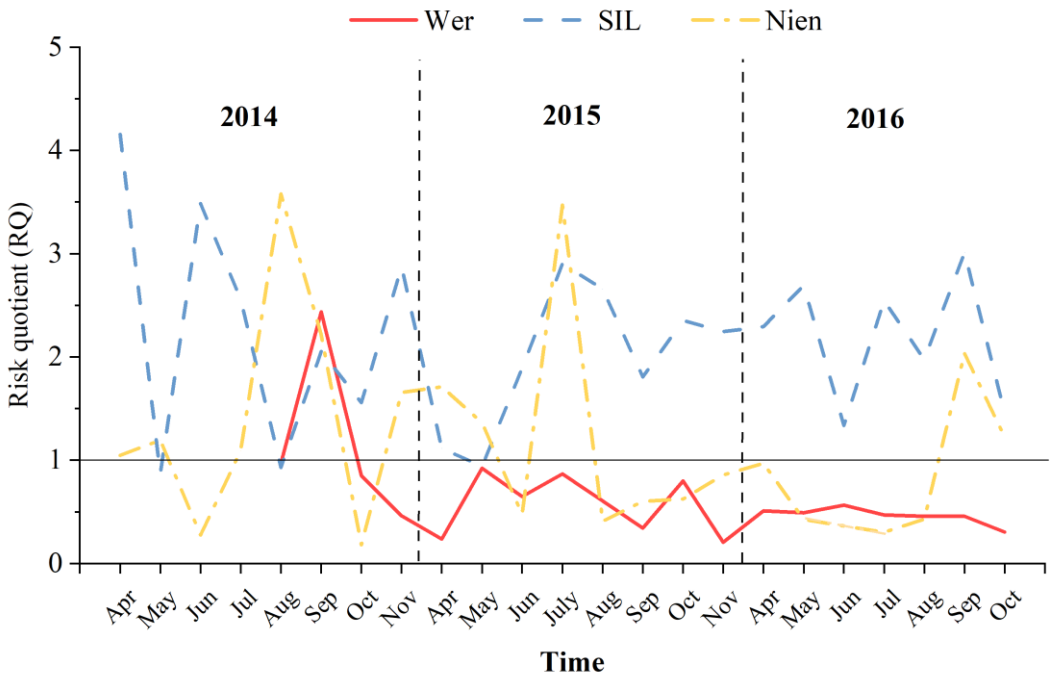


Fig. 3 Anti-AR activity from 2014 to 2016 at three sites (Wer, Sil and Nien). Water samples at the Wer site are not available until August 2014. Anti-AR activity is reported as µg of flutamide-eq. (i.e., flu-eq.) per L of water. * represents a significant difference ($p<0.05$) between the two groups. ** represents an extremely significant difference ($p<0.01$). Error bar, standard deviation (n=3). The mean anti-AR activity of all samples in a certain year represents anti-AR activity in that year.



575

576 Fig. 4 Temporal variations in the potential risk of anti-AR activity at three sites (Wer,

577 Sil and Nien).