Neural stem cell-based in vitro bioassay for the assessment of neurotoxic potential of water samples

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ABSTRACT

Intensive agriculture activities, industrialization and growing numbers of wastewater treatment plants along river banks collectively contribute to the elevated levels of neurotoxic pollutants in natural water reservoirs across Europe. We established an in vitro bioassay based upon neural stem cells isolated from the subventricular zone of the postnatal mouse to evaluate the neurotoxic potential of raw wastewater, treated sewage effluent, ground-water and drinking water. The toxic potential of water samples was evaluated employing viability, proliferation, differentiation and migration assays. We found that raw wastewater could reduce the viability and proliferation of neural stem cells, and decreased the neuronal and astrocyte differentiation, neuronal neurite growth, astrocyte growth and cell migration. Treated sewage water also showed inhibitory effects on cell proliferation and migration. Our results indicated that relatively high concentrations of nitrogenous substances, pesticides, mercuric compounds, bisphenol-A, and phthalates, along with some other pollutants in raw wastewater and treated sewage water, might be the reason for the neuroinhibitory effects of these water samples. Our model successfully predicted the neurotoxicity of water samples collected from different sources and also revealed that the incomplete removal of contaminants from wastewater can be problematic for the developing nervous system. The presented data also provides strong evidence that more effective treatments should be used to minimize the contamination of water before release into major water bodies which may be considered as water reservoirs for human usage in the future.

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Introduction

The freshwater ecosystem is a highly valuable asset for human beings. Freshwater resources such as lakes and rivers have become polluted by a large variety of chemical substances predominantly derived from anthropogenic activities. These pollutants include complex synthetic inorganic substances, heavy metals and organic substances such as pesticides, insecticides, household cleaning agents (Héritier et al., 2017; Trintinaglia et al., 2015) and pharmaceuticals (Miege et al., 2009). With a rapidly growing world population, the available freshwater resources have become scarce, so water administrative authorities are forced to manage alternative sources of water for drinking (Jia et al., 2015). Treated wastewater is now increasingly considered as a part of the common water supply (Jäger et al., 2015). Wastewater treatment plants are generally based upon conventional activated sludge and membrane bioreactor treatment techniques, which are inefficient in completely removing contaminants from wastewater (Celiz et al., 2009). The presence of hundreds of pharmaceuticals residues has been reported in effluents of wastewater treatment plants of various European and American countries (Miege et al., 2009).

Drinking water quality in Germany is coordinated through both local and international regulations, such as the Federal water act, Agenda 21 of UN (chapter-18), EU water framework directive 2000/60/EC (WRRL) and drinking water ordinances. Overall, the water supply in Germany is satisfactory. Anthropogenic factors and the decrease in summer precipitation due to climate change, however, suggest that the current water supply in Germany will reach its limits in the long term. In Germany, therefore, there is a need to utilize additional sources of water, such as the recycling of wastewater from sewage treatment plants. New advanced wastewater treatment technologies may assist in producing high-quality water that can even be employed for drinking purposes (Schmid and Bogner, 2018). The presence of high levels of pharmaceuticals and pesticides in water obtained from wastewater treatment plants in Germany reflects their inefficiency in completely removing water contaminants (Münze et al., 2017; Ternes, 1998). The Weschnitz River, which is one of the four tributaries to the Rhine River, is known to receive a huge volume of treated water from several wastewater plants in the area. High concentrations of pesticides have been reported in the water of the Weschnitz River, which is linked to sewage treatment plant effluents. Pesticide concentrations increase during the summer season because of intense agriculture activities. The Rhine River, in turn, has several catchment points for obtaining water for drinking purposes after further filtration (Quednow and Püttmann, 2007; Storck et al., 2015). To assess the quality of treated water as well as the efficacy of wastewater treatment plants, it is necessary to analyze both influents and effluents (Jäger et al., 2015).

Most of the environmental monitoring programs rely on the physicochemical characterization of water samples (Niss et al., 2018), which becomes more powerful when combined with biological testing since the latter provides deep insight into the real toxic threat of water pollutants (Zegura et al., 2009). A single kind of bioassay is insufficient to explore all kinds of toxicities associated with a complex mixture of water contaminants, so common practice employs multiple bioassays for evaluating multiple types of toxicities (Jäger et al., 2015). High total cost and ethical concerns voiced by animal welfare organizations have forced regulatory authorities to replace the traditional whole-animal-based toxicity assay systems with in vitro cytotoxicity testing (Burden et al., 2016). Major advantages of in vitro cell culture approaches include high efficacy, high reproducibility, high sensitivity, easy handling (Bianchi et al., 2015), and the ability to explore the molecular mechanism of toxicity (Poteser, 2017).

During the development of the nervous system, cell proliferation, migration, differentiation, synaptogenesis and myelination are processes that are highly sensitive to chemical exposure (Costa et al., 2008; Coullery et al., 2016). In vitro bioassay systems established for evaluation of the neurotoxicity of chemicals and water samples include zebrafish embryos, brain slices, synaptic systems, sensory systems, stem cells, primary cell models and cell lines (Hendricks and Pool, 2012; Legradi et al., 2018). Primary neural stem cells (NSCs) are basic building blocks of the nervous system. Their ease of isolation from the nervous system, ability to grow in an in vitro culture, self-renewal properties, differentiation into multiple lineages such as neurons and glial cells and capability of migrating from one location to another make NSCS a useful research tool (Bergström and Forsberg-Nilsson, 2012) for screening environmental toxicants (Tamm et al., 2006). The subventricular zone (SVZ) harbors the NSCs and represents the main site (Bollmann et al., 2014) of primary and secondary neurogenesis (Inta et al., 2008; Saha et al., 2012; Wang, 2015). Rapid proliferation and much easier handling make NSCS of SVZ a useful model for screening substances with neuromodulating properties (Liu et al., 2009).

We established an in vitro neurotoxicity bioassay based upon NSCs from SVZ of postnatal mice, to screen the neurotoxic potential of water samples on basic neurogenesis processes (viability, proliferation, differentiation and migration). Water samples were collected from a wastewater treatment plant and a drinking water distribution facility near the Weschnitz River located at two towns of the German state Baden-Wuerttemberg. Findings of this study will be highly valuable for environmental protection agencies and regulatory authorities to establish new standards for wastewater treatment plants to improve water quality.

1. Materials and methods

1.1. Water sampling

Water samples were collected from a wastewater treatment plant and drinking water distribution facilities near the Weschnitz River located in two towns of the German state Baden-Wuerttemberg. All samples were collected only once and on the same day. Sampling was performed according to the guidelines of DIN 38402-30:1998-07 (pre-treatment, homogenization and division of heterogeneous water samples) and following sampling standards: DIN 38402–11:2009-02 and DIN 38402–13:2016–09 for raw, treated and groundwater (Beuth, 2009, 2016). The sample of raw wastewater was collected from the inlet shaft before the primary clarifier,
while the sample of treated sewage water was collected from the drainage shaft after the secondary clarifier of a sewage treatment plant, Abwasserverbund Bergstraße (AVB), Altau, Weinheim, Germany. The samples of groundwater and drinking water were collected from drinking water distribution facilities, Wasserzweckverbund Badische Bergstraße, Wasserwerk, Hemsbach, Germany. The sampling cup was thoroughly washed first with 20% citric acid (NeolabMügge GmbH Heidelberg, Germany) and subsequently with distilled water before sampling. After sample collection, samples were transported in Duran glass transportation vessels which were tightly sealed. Samples were homogenized for 72 hr and then stored at 2–5°C until used for further analysis. Each sample volume was adjusted to 1 L. AVB is the biggest wastewater treatment plant in the area and receives the total sewage of seven communities in its vicinity. The AVB treats an annual wastewater volume of 12,000,000 to 18,500,000 m³/year depending on rain events and groundwater levels in the associated area. The plant processes between 20,000 and 130,000 m³ of wastewater each day depending upon the amount of precipitation (MWM, 2014). AVB plant treats wastewater by the following multiple steps: primary sedimentation, denitrification, nitrification, bio-P and sim-P.

1.2. Chemical analysis of water samples

Water samples were homogenized before chemical analysis. pH, electrical conductivity and O₂ concentration were measured using a multiparameter device (SensoN156, Hach company, USA). Ammonium nitrogen and nitrate nitrogen were analyzed by Dr HACH-Lang Cuvette Kit systems with LCK304 and LCK339 kits using a VIS-spectrophotometer (D3000, Hach company, USA) following DIN photometry protocols (DIN ISO 15923-1:2014-07, EN ISO 6978: 2004 (DEV D11)).

Pesticides N,N-Diethyl-meta-toluamide (DEET), mecoprop and terbutryn in water samples were characterized by LC-MS/MS (liquid chromatography) and dieldrin was characterized by GC-MS/MS (gas chromatography). All chromatographic analyses were carried out by an analytical laboratory (Limbach Analytics GmbH, District Court: HRB. Mannheim, Germany) with analysis reference number 17–10360 date Jul. 13, 2017. For LC-MS/MS analysis of DEET, mecoprop and terbutryn pesticides, the chromatographic separation was carried out by using a SunShell C18 CoreShell column (2.6 μm, 2.1 i.d. × 50 mm) through a Shimadzu Nexera2-HPLC system (Shimadzu, Japan) under a gradient program. Eluent A was composed of 0.1% formic acid in water and Eluent B of (0.5% formic acid and 20 mmol/L ammonium formate in methanol). The analytes were quantified by an ABSciex Triple Quad 5500-MS mass spectrometer (AB Sciex corporation, USA). For GC-MS/MS analysis, the chromatographic separation was carried out by GC column DB-35MS (30 m × 0.18 mm × 0.18 μm film) through an Agilent 7890 GC system and analytes were quantified by an Agilent 7010C Triple Quad mass spectrometer (Agilent, USA). Hydrogen gas was used as the mobile phase.

1.3. Animals

Wild-type Balb/c mice of both sexes of age 3–5 days were utilized. Animal preparations were conducted according to the guidelines of the local ethical committee and according to animal protection law in Rhineland-Palatinate, Germany.

1.4. Extraction and culture of neural stem cells from the subventricular zone of the postnatal mice

The isolation and culture of NSCs from mice SVZ for our experiments was done according to the protocol published in the literature (Bender et al., 2017). Three animals were used for each experiment. Immediately after decapitation, mice brains were removed and stored in ice-chilled MEM-medium (Life Technology, Eugene, USA) with 1% penicillin/streptomycin (ThermoFisher, Waltham, MA, USA). SVZ were dissected from both hemispheres under an inverted microscope (SZX7, Olympus, Japan) and transferred to 1 mL of HyQtase enzyme solution (HyClone-GE, USA) followed by incubation for 20 min at 37°C. Brain tissues were triturated gently using 23 gauges and 27 gage needles 3–4 times each. HyQtase was immediately removed by centrifugation of cell suspension at 100 × g for 5 min. The cell pellet was then seeded in T25 culture containing 5 mL of the proliferation medium (DMEM/F12 GlutaMax™, Life Technology, USA), 2% B-27 without vitamin A, 1% penicillin/streptomycin, ß-mercaptoethanol (Gibco, Paisley, UK), 10 ng/mL EGF and 20 ng/mL FGF (Immunotool, Germany) with the initial cell number of 500,000 to expand the cells for further experiments. Neurospheres generally appear within 3–4 days. To achieve maximum yield and cell numbers, the culture was incubated for 5 days. About half of the medium was changed every 3 days. Before starting each individual experiment, the numbers of cells were counted using the Trypan Blue method. Viability, proliferation, differentiation and migration assays were carried out by reconstituting water samples into lyophilized DMEM-F12 medium. For proliferation and viability assays, reconstituted samples were supplemented by the proliferation culture as mentioned above. For differentiation and migration assays, growth factors were omitted and the B-27 without Vitamin A was replaced by B-27 with Vitamin A (Gibco, UK). All experiments were performed in 5 replicates (n = 5).

1.5. Preparation of water samples for bioassay

A very important aspect of treating the cell-based assay with the water samples involves the adequate utilization of the water samples for medium preparation. We achieved a double-concentrated DMEM-F12 culture medium through lyophilization, which could be appropriately diluted with the water samples to be tested. The desired reconstituted medium comprising the original concentration of the ingredients of the cell culture medium was obtained with a twofold dilution of the lyophilized medium with water samples. Thus, the complete cytotoxic profile of water contaminants was achieved (Niss et al., 2018). Each water sample was initially filtered with a coarse filter followed by centrifugation (Centrifuge5–804, Eppendorf, Germany) at 20,000 r/min for 10 min to remove small visible particles. For each water sample including control, 10 mL of DMEM F-12 medium was lyophilized by freeze-drying with an Alpha 1–2 L Duplus (Fisher Bioblock Scientific, France) in a 50-mL falcon tube for about 15 hr to reduce its volume to half. The lyophilized DMEM F-12 was re-
constituted with each of the water samples so that the final volume of water sample in DMEM medium constituted 50% of the total volume. A control sample was prepared by reconstituting with double-distilled water. Other reconstituted wastewater samples were named Con for control, RWW for wastewater, TSW for treated sewage water, GW for ground water and DW for drinking water. Immediately after reconstitution of lyophilized DMEM medium with water samples, the pH of all the samples was adjusted to 7.4. Samples were filtered through a membrane filter (0.22 μm) inside the sterile bench to remove microbial contamination.

1.6. **Calcein and propidium iodide live-dead assay**

A Calcein/Propidium iodide live-dead assay was performed to evaluate the effect of water samples on the viability of NSCs. Calcein-AM (3100MP, Life Technology, USA) is a non-fluorescent compound which is readily taken up by live cells and enzymatically converted into a green fluorescent marker. Propidium iodide (Sigma-Aldrich, Germany) provides red fluorescence and serves as a marker for dead cells. Around 25,000 NSCs were expanded in reconstituted water samples supplemented by growth factor rmFGF (20 ng/mL) and rmEGF (10 ng/mL) into a 24-well plate for a period of 24 hr. At the end of the incubation period, neurospheres were formed, which were collected and dissociated enzymatically and mechanically into a single-cell suspension, followed by washing the cells three times with PBS. The cells were subsequently incubated with live and dead assay reagent containing calcein 3 μmol/L and propidium iodide 2.5 μmol/L at 37°C for 15 min. At least five independent microscopic fields were taken with the 20× lens of a fluorescent microscope (Olympus CKX41SF, Olympus Corporation, Japan) for each water sample in each replicate. Green cells were counted as live cells while red cells were counted as dead cells.

1.7. **NSC proliferation assay**

Effects of water samples on NSC proliferation were determined through the neurosphere clonogenic assay. A neurosphere clonogenic assay is especially useful in those cases where the effect on proliferation is robust. The neurosphere number count indicates the self-renewal property of NSCs, and the neurosphere diameter indicates cell proliferation within the neurosphere architecture (He et al., 2013). Clonogenic parameters, such as the number and diameter of neurospheres, are measured periodically as defined by previous researchers (Xiong et al., 2011). Around 1000 cells were seeded in 200 μL of each water sample reconstituted in DMEM F-12 proliferation medium into each well of a 96-well plate. The plate was incubated at 37°C for a total of 7 days. The medium was changed every 3 days. For clonogenic assays, the numbers and mean diameters of neurospheres for each treatment condition were counted on days 3, 5 and 7 of the culture. On each specific time point, the whole well of a 96-well plate was scanned by using the 4× objective of a microscope with phase-contrast mode. Neurosphere parameters were recorded by CELL-SENS version 1.17.

1.8. **Differentiation assay**

In vitro differentiation was performed for 7 days to quantify the neurons and astrocytes generated from NSCs (Zhang et al., 2015). NSCs were first expanded in standard DMEM F-12 proliferation medium for 5–6 days as described above. Neurospheres were dissociated into a single cell suspension followed by seeding 20,000 cells on a 12 mm glass coverslip coated with extracellular matrix (ECM) for 1 hr. After cell adherence, cells were exposed to reconstituted water samples for the following 7 days. At the end of the incubation period, cells were fixed in a solution of 4% paraformaldehyde at room temperature for 20 min followed by washing with PBS three times. The fixed cultures were processed further for immunostaining.

1.9. **Migration assay**

NSCs were first proliferated in standard DMEM F-12 proliferation medium supplemented with growth factors, for 5–6 days. Then 15–20 neurospheres were allowed to attach at the surface of a 12 mm glass coverslip coated with Poly-D-lysine (10 μg/mL) and incubated in a 24 well plate for 1 hr. placed in an incubator at 37°C. Attached neurospheres were exposed to reconstituted water samples for the next 24 hr. Phase-contrast photographs of each well were taken by a microscope using a 4× objective. Measurements were performed according to the protocol described by previous researchers (Baumann et al., 2014). Briefly, four radii of each differentiated neurosphere were calculated by measuring the distance travelled by migrated cells at a right angle to the edge of a neurosphere core from all four directions (Fig. 5). At least 15 neurospheres were included for each replicate per each condition.

1.10. **Immunostaining**

Differentiated fixed cell cultures were immunostained for the neuronal and astrocyte markers. Staining was performed by following a published protocol with slight changes (Bernas et al., 2017). Immediately after differentiation, cells were fixed with 4% paraformaldehyde solution (Sigma-Aldrich, Germany) for 30 min. Cell permeability was enhanced by incubating cells with a solution of Triton 100× (0.3%) for 10 min at room temperature followed by washing with PBS-tween once and with PBS twice. Cell surfaces were blocked by incubating them with 10% Normal donkey serum (Merck, Germany) for 1 hr at room temperature. Cultures were subsequently incubated at room temperature for 1 hr. with the following primary antibody solutions in PBS with 5% blocking agent: (mouse-anti β-tubulin III, 1:500) (MAB1637, Merck, Germany) and (rabbit anti-GFAP, 1:500) (Z0334, Dako, Denmark). The cultures were subsequently washed thrice with PBS. Cultures were then further incubated with Alexafluor 488 & Alexafluor 594 conjugated donkey antibodies (Life technology, USA) for 1 hr, and finally washed thrice with PBS. Finally, the nuclei of cells in culture were counterstained by DAPI (1:500) (Sigma-Aldrich, Germany) for 10 min then finally washed with PBS. The fluorescent mounting medium was used to fix coverslips on glass sides (Ostenfeld and Svendsen, 2004).
### 1.11. Statistical analysis

Data were analyzed statistically using descriptive statistics and the Kruskal Wallis test with the post hoc Dunn's test. Data were accepted as statistically significant under a probability range of 5%.

### 2. Results

#### 2.1. Physicochemical characterization of water samples

Table 1 Represents the general quality parameters of the water samples. Both RW and TSW contained higher concentrations of ammonium-and nitrate-nitrogen when compared to the GW and DW, while electrical conductivity values were lower in the case of RW and TSW. The quantitative analysis of the pesticides revealed that dieldrin was present under the safe limit in all water samples. DEET and terbutryn were not detected, whilst mecoprop was found within the safe limit in DW. The concentrations of both DEET and terbutryn were found to be elevated in RW and TSW. All four pesticides were detected in GW, but none of them was above the safe limit, see Table 2. Chromatograms of dieldrin, DEET, terbutryn and mecoprop pesticides are given in Appendix A Figs. S13-S14.

#### 2.2. NSC-based bioassay

We initially performed baseline studies to perceive if the lyophilization and subsequent reconstitution of DMEM/F-12 medium with water samples adversely affect the medium quality. In baseline studies we performed viability, proliferation, and differentiation assays for normal DMEM/F-12 and lyophilized DMEM/F-12 reconstituted with double-distilled water. Our baseline study showed insignificant difference between normal DMEM/F-12 and reconstituted DMEM/F-12 in terms of viability, proliferation, differentiation and migration of NSC culture (Appendix A Figs. S13-S14).

#### 2.2.1. Viability and proliferation assay

The Calcein-AM/PI live-dead assay is widely used to determine the toxic effects of chemical compounds in mammalian cells, and a previous researcher employed this assay to determine the effects of tap water contaminants on the viability of water flea, a bio-indicator for water toxicity (Teplova et al., 2010). We performed Calcein-AM/propidium iodide live-dead assays to evaluate the acute toxicity of the water samples on NSCs when exposed for 24 hr. The results demonstrated that RW significantly reduced the percentage of living cells when compared to the Con (Fig. 1a). The neurosphere clonogenic assay was performed to evaluate the effect of water samples on NSC proliferation. RW significantly reduced the count and mean diameter of neurospheres (Fig. 1b, c and Fig. 2) when compared to the Con and all other treatment conditions at all observation time points. A significant difference between RW and TSW was, however, only observed on day 3. We also observed a significant difference between the number and mean diameter of TSW vs. Con and TSW vs. GW only on day 7.

#### 2.2.2. Differentiation assay

We carried out a differentiation assay to evaluate the effect of water samples on the multipotency of NSCs. Differentiated cells were identified by immunostaining of β-tubulin III as a neuronal marker, and GFAP as an astrocyte marker. In the differentiation experiment, the reason for focusing only on differentiated neurons and astrocytes was their pivotal role in the development and functions of a nervous system and high vulnerability to toxic insult. Additionally, the neuronal/astrocyte co-culture system is a proven in vitro

### Table 1 – General quality parameters of water samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RW</th>
<th>TSW</th>
<th>GW</th>
<th>DW</th>
<th>Safety limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color pH</td>
<td>Black gray 7.8</td>
<td>Colorless 6.2</td>
<td>Colorless 6.7</td>
<td>Colorless 7.2</td>
<td>Colorless 6.5–8.5</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>261</td>
<td>716</td>
<td>822</td>
<td>1013</td>
<td>2500</td>
</tr>
<tr>
<td>O₂ concentration (mg/L)</td>
<td>0.78</td>
<td>6.74</td>
<td>2.86</td>
<td>5.13</td>
<td>5.0</td>
</tr>
<tr>
<td>NH₄⁺ (mg/L)</td>
<td>124.6</td>
<td>0.68</td>
<td>0.409</td>
<td>0.004</td>
<td>0.5</td>
</tr>
<tr>
<td>NO₃⁻ (mg/L)</td>
<td>1.13</td>
<td>8.600</td>
<td>0.111</td>
<td>0.145</td>
<td>50</td>
</tr>
</tbody>
</table>

RWW: raw wastewater; TSW: treated sewage water; GW: groundwater; DW: drinking water; N/A: not available. Safety limit for drinking water (Organization, 2017).

### Table 2 – Pesticide traces in water samples.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>RW</th>
<th>TSW</th>
<th>GW</th>
<th>DW</th>
<th>Safety limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dieldrin (μg/L)</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>Undetectable</td>
<td>0.03</td>
</tr>
<tr>
<td>Diethyltoluamid (μg/L)</td>
<td>0.75</td>
<td>0.13</td>
<td>-0.01</td>
<td>Undetectable</td>
<td>0.10</td>
</tr>
<tr>
<td>Mecoprop (μg/L)</td>
<td>0.03</td>
<td>0.14</td>
<td>-0.01</td>
<td>Undetectable</td>
<td>0.10</td>
</tr>
<tr>
<td>Terbutryn (μg/L)</td>
<td>0.18</td>
<td>0.19</td>
<td>0.02</td>
<td>Undetectable</td>
<td>0.03</td>
</tr>
</tbody>
</table>

model and has been used extensively in toxicology studies (Anderl et al., 2009; De Simone et al., 2017; Deng and Poretz, 2003; Jiang et al., 2015). Percentages of differentiated cells and neurite outgrowth and the percentage of each glass coverslip area covered by differentiated cells were calculated using CELL-SENS and image J software. RWW significantly reduced the percentage of neurons and astrocytes and significantly increased the percentage of double-negatives (cells non-reactive to both neuronal and astrocyte markers) when compared to the Con and other water treatments (Fig. 3a). The strongest difference in the percentage of neurons was observed between RWW and DW (37%), whilst the percentage of astrocytes differed significantly between RWW and GW (26%). Similar trends with respect to the percentage of area covered by differentiated neurons and astrocytes cells in each microscopic field were observed for RWW and all other water treatments (Fig. 3b). RWW strongly inhibited the earlier neurite growth and hence gave rise to the highest percentage of neuriteless neurons (67% of total neurons) when compared to all other water treatments. Concerning the percentage of neuriteless neurons, the highest difference of around 95% was calculated between RWW vs. Con and RWW vs. DW (Fig. 3c). The morphological features of differentiated neurons and astrocytes under different water treatments are shown in the Fig. 4. Neurons were well-grown with extended neurites and multiple branches when exposed to Con, GW, and DW. In contrast, the cells exposed to RWW were observed to be detached at the end of observation period, and the ones that survived demonstrated highly depressed growth, with intermingled deformed neurites without a well-developed branching pattern. Similarly, cells exposed to TSW also demonstrated depressed neuronal growth, with less dense neurites when compared to other water treatments. A leaf-like morphology of astrocytes was observed for all samples except RWW, which demonstrated depressed growth with elongated thread-like morphology.

2.2.3. Migration assay
The nerve sheath migration assay is a very strong and relevant tool for screening the effect of environmental pollutants on neural cell migration in developmental neurotoxicity studies. The assay effectively mimics the in vivo cell migration process (Fritsche et al., 2011). The nerve sheath migration assay was performed to measure cell migration under the influence of water samples for 24 hr, and calculations were done using CELL-SENS software. RWW significantly reduced the mean migration distance travelled by the differentiated cells when compared to the Con, GW and DW. A significant difference concerning the mean distance travelled by differentiated cells was also calculated between Con and TSW. The strongest difference was calculated between Con vs. RWW (34%) and RWW vs. DW 33% (Fig. 5).

3. Discussion
Several ecological studies identified industrial discharges and inefficiency in wastewater treatment techniques as two main sources for high concentrations of cytotoxic and neurotoxic
pollutants present both in wastewater and treated wastewater (Fricke et al., 2015; Fritsche et al., 2018; Gerhardt, 2019; Héritier et al., 2017; Karlsson et al., 2020; Le et al., 2017; Miege et al., 2009; Poteser, 2017; Trintinaglia et al., 2015). NSC is an attractive and robust cell-based in vitro model to investigate the neurotoxicity of toxic substances. An in vitro assay based upon NSC was successfully employed to explore the neurotoxicity of tap water samples (with nickel pollutant) on neuroproliferation and neuronal differentiation (Zhou et al., 2019). In the present study, we developed an in vitro assay employing NSCs isolated from SVZ of postnatal Balb/c mice to assess the neurotoxicity of wastewater, effluent from the wastewater treatment plant, groundwater and drinking water. We selected lyophilized DMEN/F-12 medium reconstituted by double-distilled water as a control for the rest of our assays. Our baseline study showed that the lyophilization and reconstitution processes did not adversely affect the DEME/F-12 media quality for NSC culture.
3.1. Physicochemical characterization of water samples

Our results demonstrated that the levels of nitrogenous contaminants and pesticides were below the safe limit in GW and DW. RWW and TSW contained relatively higher concentrations of ammonium compounds, nitrates, pesticides DEET, terbutryn and mecoprop. Wastewater treatment plants working in some EU countries are unable to completely remove pollutants from raw wastewater. A study conducted in Spain reported that effluents of three different wastewater treatment plants contained higher concentrations of pesticides as compared to the untreated raw wastewater. The presence of relatively higher concentrations of pollutants in the effluents of wastewater treatment plants was due to the release of pollutants from the plants themselves (Köck-Schulmeyer et al., 2013). The presence of high nitrate contents in water is a product of highly decayed plant contents, usage of animal manure and leakage of septic tank effluents, and excessive use of fertilizers (Huang et al., 2018; Manassaram et al., 2005). High ammonium levels in wastewater (Seruga et al., 2019) and wastewater treatment plant effluents have been recently reported (Huang et al., 2018). An excessive amount of nitrogenous contaminants in treated wastewater reflects the inefficiency of the wastewater treatment process (Santos et al., 2008). Periconceptional exposure of human mothers to a high concentration of nitrate through drinking water resulted in neural tube defects in the fetus and anencephaly in newborns (Croen et al., 2001) and neural tube defects in zebrafish embryo by disrupting estrogen receptors (Jannat et al., 2014).

In Germany, a high concentration of DEET was reported in water samples collected from different sources. The Danube river in German territory, for instance, which receives a huge amount of water from widely dispersed wastewater treatment plants in the area (Loos et al., 2017), was reported to contain a high concentration of DEET in untreated wastewater and effluents of wastewater treatment plants (Launay et al., 2013), in surface water and wastewater influents (Aronson et al., 2012). DEET is an environmental toxicant whose toxic concentration has been detected in marine water near nuclear power plants. DEET is considered toxic and neurotoxic (Abou-Donia et al., 1996; de Assis Martini et al., 2017). Terbutryn is a commonly employed herbicide and an environmental toxicant, with high concentrations detected in drinking water and processed food in several countries (Villarini et al., 2000). A high concentration of terbutryn was reported in surface water of the Llobregat River near Catalonia, Spain (Rubírola et al., 2019). In another study conducted in Germany, toxic levels of terbutryn were not only detected in untreated raw water but also in effluents of wastewater treatment plants (Le et al., 2017). A European study revealed the presence of DEET and terbutryn at high concentrations in most of the effluents tested (Loos et al., 2013). In Sweden and Denmark, high concentrations of mecoprop pesticide were detected both in untreated water and wastewater treatment plant effluents (Bollmann et al., 2014). Mecoprop was also detected in stream water in the German countryside. However, the concentration of mecoprop was below the safe limit (Schulte-Oeflmann et al., 2011). A high concentration of mecoprop was reported in groundwater collected from Weaver and Gowy catchments in the UK. The concentration

**Fig. 3** – NSC differentiation after 7 days of incubation with water samples. Percentage of neurons and astrocytes was calculated from total DAPI +ve nuclei count. Area of 12 mm glass coverslip covered by differentiated cells was calculated from 4x4 mosaic pictures taken with 4x objective of a fluorescent microscope. (a) Represents the percentage of neurons, astrocytes and the cell which were non-immunoreactive for both neuronal and astrocyte cell markers. (b) Represents the percentage of area covered by differentiated cells on each coverslip. (c) Indicates the percentage of neuriteless neurons (NLN) calculated from the total neuronal count. Values are mean ± SEM of 5 independent replicates (n = 5). *p < 0.05, **p < 0.01 and ***p < 0.001.
detected was higher than EU drinking water quality standards (Idowu et al., 2014). The presence of dieldrin in wastewater treatment plant effluents (Kenny et al., 2017), marine surface water and in potable water was also reported by researchers (Díaz-Barriga Arceo et al., 2015; Kenny et al., 2017).

3.2. Effects on viability

Only RWW significantly reduced the percentage of viable cells when compared to the Con. A study conducted in Slovenia reported on the cytotoxicity of untreated wastewater and wastewater treatment plant effluents in different dilutions toward a human hepatoma cell line (Zegura et al., 2009). Severe neurotoxicity in fish upon exposure to diluted effluent from a wastewater treatment plant was reported in Noksan (South-Korean) due to high concentrations of pesticides and industrial compounds that were not removed effectively by the wastewater treatment plant (Park et al., 2009). The possible reason for the inhibitory effects of RWW on NSC viability is the complex combination of pollutants derived from anthropogenic activities whose release was not controlled. Polycyclic aromatic hydrocarbons (Tang et al., 2003), phthalates (Lim et al., 2009), methyl mercury (Farina et al., 2011) and pesticides (Lin et al., 2018) are notorious for their neurotoxicity. High concentrations of polycyclic aromatic hydrocarbons were reported in the Neckar River in Southern Germany (Vincze et al., 2015). Moreover, elevated levels of phthalate in municipal wastewater (Fromme et al., 2002), methyl mercury in different lakes and rivers (Euractive, 2018) and pesticides such as atrazine, terbutylazine, metazachlor in different water beds in Germany (Karlsson et al., 2020) were also observed. A high concentration of ammonium compounds in RWW may contribute to neurotoxicity since an excess of ammonium compounds induces cytotoxicity in murine myeloma cells (Martinelle and Hägström, 1993). The literature also reported the neurotoxicity of DEET (Swale et al., 2014) and cytotoxicity of terbutryn (Villarini et al., 2000). The role of these pesticides in the neurotoxicity of RWW is not clearly understood, since the levels employed in published studies were several times higher than those detected in our RWW and TSW samples. Poor neural cell viability in children due to neurotoxicity induced by environmental pollutants during the early stage of life results in neurodevelopmental disorders such as defective locomotive functions, weak memory (Tseng et al., 2013) and poor IQ scores (Gorini et al., 2014). Our results indicated that wastewater treatment plants sufficiently reduced the toxic potential of pollutants in the studied water samples.

3.3. Effect on cell proliferation

Counting the number and diameter of neurospheres at specific time intervals provides an estimation of NSC proliferation capability (Lu and Wong, 2005). Our results demonstrated that both RWW and TSW inhibited NSC proliferation. RWW inhibited proliferation at all observation time points, whilst TSW inhibited proliferation only at later stages of incubation when compared to the Con and GW. Our findings are consistent with a published study showing that both the raw wastewater influent and treated effluents significantly reduced the proliferation of a human embryonic kidney cell line by altering the expression of cell cycle regulatory proteins, due to the presence of multiple contaminants in the water samples (Ren et al., 2017). Untreated sewage effluent contained pharmaceutical contaminants including atenolol, caffeine, hypnotics and antihypertensive drugs, and was reported to strongly inhibit cell proliferation in the brain of Prochilodus lineatus (Pérez et al., 2018). The decreased NSC proliferation with RWW and TSW could be attributed to the presence of one or more environmental pollutants with potential cell proliferation inhibition properties. Inhibitory effects of organic mercury compounds (Bose et al., 2012), bisphenol-A (Tiwari et al., 2015), the pesticide DEET (Parihar et al., 2013), and nitrates (Solari et al., 2009) on the proliferation of NSCs and effects of pharmaceutical contaminants on fish brain cells (Pérez et al., 2018) at ultra-low doses were reported by researchers. Moreover, the literature reveals that a high amount of methyl mercury has been found in a large number of lakes and surface water deposits across Europe, including Germany (Euractive, 2018). DEET pesticide was found in elevated concentrations in the Danube river (Loos et al., 2017), treated and untreated wastewater (Launay et al., 2013) and in surface water (Aronson et al., 2012). A very high concentration of bisphenol-A was also observed both in treated and untreated wastewater in different cities of Germany (Fromme et al., 2002). Decreased proliferation of NSCs in the brain is linked to poor memory, learning dysfunction (Parihar et al., 2013) and schizophrenia at early ages in children (Reif et al., 2006). Future studies need to conduct dose-response assays for pure DEET and nitrates since both pollutants were found in high concentrations in RWW and TSW, especially when these pollutants pose inhibitory effects on NSC proliferation. Furthermore, our findings demonstrated that wastewater treatment plants did not remove the pollutants completely, resulting in the inhibitory effects of TSW on cell proliferation.

3.4. Effect on differentiation

RWW significantly decreased the percentage of neurons, astrocytes, neuronal growth area, and astrocyte growth area, and increased the percentage of neuriteless neurons when compared to the control. Although no significant difference was observed between Con vs.TSW, yet TSW depressed the neurite growth and elongated the astrocytes when compared to Con, GW, and DW (Fig. 3). TSW also exhibited neutralizing effects since areas covered by differentiated neurons and astrocytes were significantly higher than those with RWW treatment. Many environmental pollutants frequently detected in treated and untreated water bodies of European countries were studied for their effect on neuronal and astrocytes differentiation of NSCs. Ammonium compounds (Braissant et al., 2002), nitrates (Solari et al., 2009), methyl mercury (Tamm et al., 2006), and bisphenol-A (Fujiwara et al., 2018; Tiwari et al., 2015) were reported to have inhibitory effects on neuronal differentiation, whilst artificial sweeteners (Cong et al., 2013), the pesticide DEET (Christen et al., 2017), Dieldrin (Richardson et al., 2006) and phthalates (You et al., 2018) were reported to exhibit inhibitory effects on neurite growth. Pizzurro et al. (2014) in their study reported the inhibitory effects of diazinon pesticide on astrocyte differentiation and development. The inhibitory ef-
effects of these pollutants on neuronal and glial differentiation were evaluated at very low dose levels. Studies reported the presence of organic mercury compounds (Euractive, 2018; Fricke et al., 2015), bisphenol-A (Gerhardt, 2019), artificial sweeteners (Scheurer et al., 2009), the pesticide DEET (Loos et al., 2017) and phthalates (Fromme et al., 2002) at high levels both in untreated raw water and treated water samples collected from different locations in Germany. We speculate that besides other contaminants in RWW, DEET also contributed to the inhibitory effects on neurodifferentiation. Poor neurite growth is implicated in autism (Gilbert and Man, 2017), and poor astrocyte development is linked to mood disorders (Koyama, 2015) in human beings. In future studies, it is very important to investigate the neuro-inhibitory effects of DEET and dieldrin at concentrations we detected in RWW and TSW. Dieldrin was found at very low concentration, yet its synerg-
Neurosphere migration assay for determination of the effect of water samples on cell migration. Migration assay was performed in a 24-well plate. Phase-contrast pictures of neurospheres were taken 24 hr after incubation with 10× objective of a phase-contrast microscope. The yellow dotted line in images encircles the area occupied by migrated cells and the red dotted line encloses the neurosphere core. White arrows in the method image indicate the distance travelled by migrating cells away from the edge of the neurosphere core. Bar graph represents the mean distance travelled by migrating cells in four directions for each treatment condition. Values are presented as mean ± SEM of 5 independent replicates (n = 5). *p ≤ 0.05, **p < 0.01 and ***p < 0.001, ****p < 0.0001.

Tic effects with other complex mixture of pollutants must not be ignored, since chemical interaction between environmental pollutants can result in an increase or decrease in the toxicity of an individual compound (Krishnan and Brodeur, 1994). One such type of interaction was reported in the literature in which dieldrin synergistically interacted with H₂O₂ to cause severe toxicity in rat thymocytes (Chimeddorj et al., 2013).
3.5. Effect on cell migration

Cell migration is an important subset of the neurogenesis process. In the event of brain trauma or injury, SVZ is the first area where NSC stem cell proliferation and migration take place to repair the injured tissues (Galindo et al., 2018). Both RWW and TSW inhibited cell migration when compared to Con and DW. Many environmental toxicants, such as organic mercury compounds, steroidal drugs, the environmental toxicant bisphenol-A and pesticides, inhibit the migration of cells in the nervous system (Fabrión et al., 2012; Ishido and Suzuki, 2010a, 2010b). The occurrence of these environmental toxicants was reported both in wastewater and wastewater treatment plant effluents of different countries of the world (Gbondo-Tugbawa et al., 2010; Mohapatra et al., 2011; Pauwels et al., 2008), including Germany (Aronson et al., 2012; Euractive, 2018; Gerhardt, 2019). Although we could not find any published study to directly support our findings, we assume that the inhibitory effects of RWW and TSW on cell migration might be due to the presence of many environmental toxicants in our samples that were not completely removed by the water treatment plant. A decrease in neural cell migration increases the risk of epilepsy in human beings (Stouffer et al., 2016).

Although neurotoxicity of DEET and mecoprop in human beings (Petrucci and Sardini, 2000; Wiles et al., 2014), and teratogenic effects of terbutryn in an animal model (Meulenbelt et al., 1988; Velisek et al., 2012) at very low dose levels were reported in the literature, data regarding the neurotoxic concentrations of these pesticides in living body fluids is lacking. Prospective researchers are suggested to conduct biotransformation studies parallel to dose-response toxicity studies of DEET, terbutryn and mecoprop pesticides.

4. Conclusions

Our results demonstrated that groundwater and drinking water presented no sign of inhibitory effects on any of the neurogenerative processes (viability, proliferation, differentiation and migration) in an in vitro neurotoxicity assay based upon NSCs from SVZ of postnatal mice. Raw wastewater inhibited all neurogenesis processes, while treated sewage water showed inhibitory effects only on proliferation and migration. Through this study, we confirmed that an in vitro NSC-based assay provides a highly sensitive and robust system for neurotoxicity screening of diluted water samples from different sources with different levels of contamination. The assay also unveiled the tendency of treated sewage water to inhibit the differentiation of NSCs. Finally, it can be concluded that NSC-based in vitro assays offer a very good platform for screening water pollutants with neurotoxic potential, and should be considered as an integral part of other bioassays for evaluating the quality of water samples containing a mixture of chemical pollutants with different modes of action before these water samples are released into the environment or considered for human consumption. These findings also reflect the inefficiency of wastewater treatment plants in the studied area in completely removing the toxic pollutants and direct the attention of water management authorities to review their protocols regarding wastewater treatment practices.

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Appendix A. Supplementary data


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