Influence factors of organic compounds leaching from PE pipes and the potential toxic effects on E. coli and rat C6 glioma cell

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ABSTRACT

Water quality safety is always a hot topic. Recently there has been a great increase in the use of polyethylene (PE) pipes in drinking water distribution systems in China. Some organics can leach from PE pipes into drinking water, which is undoubtedly harmful for consumers. In this study, potential organics leaching from two Chinese brands of PE pipe were investigated in order to determine the potential risk to both consumers and water quality. Their released amounts are characterized by UV$_{254}$ instead of total organic carbon (TOC). Organics are identified by means of gas chromatography coupled with mass spectrometry (GC-MS). Influencing factors such as types of water, time, pH, residual chlorine are discussed. The cytotoxicity of leaching organics is first put forward in this study, and rat C6 glioma cell is used to assess the effect of leaching organics; the increasing dosage of soaking solution negatively affects morphology and relative viability of rat C6 glioma cell. The results show that residual chlorine, time and temperature have a positive effect on organics release, while pH plays an opposite role.

Key words | cytotoxicity, drinking water, influence factors, PE pipes, released organic compounds

INTRODUCTION

In recent years, there has been a great increase in use of polyethylene (PE) pipes in drinking water distribution systems (DWDS) and household installations. Compared with iron pipes, plastic pipes are easily installed and cut, and are resistant to corrosion, and tolerant to slight displacement (Kowalska et al. 2011). However, the microorganism and chemicals in drinking water may let organic compounds leach from PE pipes (Nahar et al. 2011) and cause water contamination. PE materials are potentially capable of leaching monomers such as antioxidants, lubricants, softeners, coloring agents, applied solvents (Tomboulian et al. 2004) and plasticizer (Nahar et al. 2011). Such leaching chemicals can occur directly by diffusing chemicals into the water, or indirectly by turning into by-products or impurities, which result from the synthesis of pure phenolic additives, before leaching into water (Brocca et al. 2002).

Many studies have been performed about leaching organic compounds. Lund et al. (2011) investigated migration of volatile organic compounds (VOCs) from cross-linked polyethylene (PEX) pipes produced by different production methods. It is reported that phenols and quinones were found in water exposed to high density polyethylene (HDPE) pipes or PE pipes (Brocca et al. 2002; Skjevrak et al. 2003; Kowalska et al. 2011). Koch (2004) has found about 100 different organics leaching from PE and PEX pipes. Lee (2013) has measured released amounts of VOCs in five types of plastic pipes (polybutene (PB), polyvinyl chloride (PVC), polypropylene (PP), PE and chlorinated polyvinyl chloride (cPVC)).

Some research has been carried out on the influencing factors of organic carbon leaching. Walter et al. (2011)
studied the effects of biofilm, pipe age and diameter on PVC and cPVC pipes releasing vinyl chloride monomers (VCMs), and discovered that biofilm has a positive effect as well as a negative effect on VCMs release. In another study (Yu et al. 2011), the depletion of antioxidant system occurred more rapidly with water containing chlorine dioxide than chlorinated. Al-Malack et al. (2000) reported that VCMs leaching from unplasticized polyvinyl chloride (uPVC) pipes are affected by time, temperature, pH and total dissolved solids; uPVC pipes only released VCMs when the water temperature was higher than 35 °C. Maria et al. (2011) have studied the effect of different weathering conditions (a combined action of ultraviolet, time and relative humidity) on PE pipes releasing phenolic antioxidant. In our study, type of water and concentration of disinfectant are also considered as influencing factors.

To characterize the released amount of organic compounds, total organic carbon (TOC) has been used in previous research. But UV254 has more rationality than TOC, because it is proportional to the concentration of aromatic compounds (Kelley et al. 2014), and humic substances and organic compounds, which contain C=C bonds and C=O bonds, UV254 has been used as a substitute for dissolved organic carbon (DOC) in water (Chow et al. 2008). It is also reported that the UV254 value has some relation to TOC, DOC and chemical oxygen demand (COD) (Eaton 1995), which reflect the degree of organic pollution. Therefore, it can indirectly represent the concentration of a class of organic compounds which are aromatic compounds or humic substances or which have the same double covalent bonds (C=C bond and C=O bond). In addition, UV254 is a simpler and more feasible index, which has universal significance as a new comprehensive index of organic pollution.

Although in previous works, it is mentioned that leaching organic compounds do affect human health, there is little research about cytotoxicity of leaching organics and its effect on cell growth. In order to assess the risk of organic compounds, a microbial culture and cell toxicity tests were carried out. Escherichia coli was used as a model microorganism to study the effect of organic compounds on the growth of microorganisms. Rat C6 glioma cells (hereinafter referred to as C6 cells) were used to study the cytotoxicity of leaching organic compounds, and the preliminary quantitative conclusions regarding health risk are put forward in this study.

**MATERIALS AND METHODS**

PE pipes (brands A and B) which were used in this study were bought from the manufacturers in China. Tap water was from Hangzhou (a Chinese city whose maximum daily water supply is 1.4 million tons), ultrapure water was prepared with Milli-Q apparatus (Millipore, Molsheim, Germany). Soaking solution was prepared according to Standard for safety evaluation of equipment and protective materials in drinking water system (1998).

In the sample pretreatment procedure, pipe sections with a length of 0.5 m were flushed for 1 h and kept in contact with ultrapure water in stagnant conditions for 24 h. The residual chlorine solution at different concentrations (0, 0.5, 1.0, 1.5 and 2 mg/L) was prepared also according to GB/T 17219-1998. Test tubes were filled with different concentrations of chlorine solution for 72 h, reference samples were prepared by storing different concentrations of residual chlorine in glass bottles. In the experiments about other influencing factors: test tubes were filled with different test water for 24 h, 72 h, 144 h and 216 h respectively, reference samples were prepared by different test waters in glass bottles; test tubes were filled with ultrapure water at different pH values for 72 h, reference samples were prepared by storing different pH ultrapure waters in glass bottles; soaking solution was poured into the test tubes under a controlled temperature at 5, 10, 15, 20, 25 and 30 °C for 72 h respectively, reference samples were prepared by storing soaking solution under different temperature in glass bottles. At the end of each test period, water samples were extracted to detect by ultraviolet–visible spectrophotometer (UV-Vis). The ΔUV254 value represents the concentration of a class of organic compounds which are leaching from the PE pipe, as expressed by Equation (1):

\[
\Delta UV_{254} = UV_{254\, sample} - UV_{254\, reference}
\] (1)

The kinetics of bacterial growth in the soaking solution of PE pipes have been studied. The microbial experimental procedure is depicted in Figure S3 (available with the online version of this paper). After preparing control samples, Escherichia coli (E. coli, 100 μL) was cultured in 25 mL soaking solution at 37 °C with continuous agitation for 0 h, 2 h, 5 h, 8 h, 12 h, 15 h and 18 h, respectively.
Optical density (OD) value at 600 nm was determined by UV-Vis. The growth rate is expressed by Equation (2):

\[
\text{Growth rate} = \frac{OD_{600 \text{ sample}} - OD_{600 \text{ control}}}{OD_{600 \text{ control}}} \times 100\% \tag{2}
\]

The cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction experiment. C6 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (200 μL/well) in a 96-well plate for approximately 16 h under the conditions 37 °C, 5% CO2 atmosphere. The culture medium was discarded and the cells washed with phosphate-buffered saline (PBS). MTT, PBS (10 μL, 5 mg/mL) and RPMI 1640 medium without FBS (90 mL) were added to each C6 cell sample, and incubated for another 3 h in 37 °C, 5% CO2 atmosphere after exposing C6 cells to solid phase extraction (SPE) solution (6, 16, 33, 66 and 100 μL/well) for 3 h. Supernatants were removed, and dark blue formazan crystals were dissolved in 100 mL dimethyl sulfoxide (DMSO). After leaving the samples at room temperature for 10 min, the absorbances of the samples were recorded at 570 nm with a microplate reader. Simultaneously, control samples and contrast samples were prepared. Each experiment was performed at least in triplicate. The relative cell viability is expressed as Equation (3):

\[
\text{Relative cell viability} = \frac{OD_{\text{sample}} - OD_{\text{control}}}{OD_{\text{contrast}} - OD_{\text{control}}} \times 100\% \tag{3}
\]

The organic compounds have been identified by gas chromatography coupled with mass spectrometry (GC-MS). Relevant parameters for GC-MS, details about materials, colony counting method and SPE method can be found in the supplementary information (available with the online version of this paper).

RESULTS AND DISCUSSION

Influence factors

Releasing amount of different kinds of water samples follows the order: tap water > soaking water > ultrapure water (Figure 1(a)). The reasons may mainly be: (1) the conductivity of ultrapure water is less than 2 μs/cm and no substance has been added; (2) soaking solution has calcium ions, sodium ions and residual chlorine, which may affect release amount; and (3) tap water has different pHs and many compounds such as disinfection by-products, which may affect release rate. Therefore, pH, residual chlorine, disinfection by-products and inorganic ions may affect the migration of organic compounds from PE pipe into water.

The ΔUV254 value gradually increases with the increasing concentration of residual chlorine (Figure 1(b)). Long-term exposure to residual chlorine was known to have a deleterious effect on the mechanical characteristics of PE pipe. It is reported that contaminants degradation in PE pipe occurred after antioxidants were depleted (Gedde et al. 1997). The depletion occured non-uniformly due to natural antioxidant migration from PE pipes and simultaneous penetration and consumption of chlorine. ΔUV254 increased rapidly between 0 and 0.5 mg/L, but varied very little between 0.5 and 1.5 mg/L, then presented a rising tendency when the concentration of residual chlorine was over 1.5 mg/L. A possible reason for this phenomenon is that when additive dosage of disinfectant increases, extra residual chlorine reacts with antioxidants contained in PE pipes with no obvious rise of the concentration of organic compounds. Then the released amount increases rapidly with increasing residual chlorine.

pH is another critical parameter because it causes chlorine to accelerate pipe age. pH values of drinking water are occasionally between the range of 5 to 8.5, so pH values were discussed from 5.0 to 9.0 in this study. Release amount of organic compounds varied obviously and showed a decreasing trend with increasing pH value (Figure 1(c)), which means that weak acidic condition has a relatively strong erosion effect on PE pipes. Al-Malack et al. (2000) also got a similar result when he studied the effect of pH on the release amount of VCMs leaching from uPVC pipes. His explanation was that acidic water strongly eroded the plastic pipe surface. Release amount reached a steady state under neutral conditions (pH = 7 ± 0.5), which corresponds to Kowalska’s research (2013). Acidic conditions will accelerate aging of PE pipe, leading to promotion of the release of organic compounds. In
China, chlorine is widely used in drinking water disinfection, and is highly dependent on the acidic environment. Hence, it is very important that pH value be controlled in DWDS.

Temperature was the most important factor in influencing the diffusion rate. The released amount was quite small between 5 and 15 °C, and then increased significantly between 15 and 30 °C, which was 4.267 times more than the effect of 15 °C (Figure 1(d)). The result indicated that the glass transition temperature of polymers had profoundly influenced the transport properties. Glass transition temperature is one of the polymer property indices, it represents the transition temperature at which the polymer turns into the elastomeric state from the glassy state, and vice versa. Polymers with lower glass transition temperature will have higher diffusivity (George & Thomas 2001).

Soaking time also has significant influence on organics release. It reached 95% and 95.12% on brands A and B for 3 days, respectively. Release rate decreases during 3–30 days to 29.2% and 8.89% for brands A and B, respectively. Nevertheless, release amount increased again from 30 to 60 days (Figure S1, available with the online version of this paper). In addition, the productive process of PE pipes affects the internal decomposition of components and migration. Release rate is not simply decreasing or increasing during the migration period, on the contrary there exists a certain degree of fluctuation, which is a big problem for evaluation of the material security (Wang et al. 2009).

GC-MS analysis

GC-MS analysis was carried out in order to further identify organic compounds leaching from PE pipes.
The real time of peaks (a) – (f) are 14.91 min, 28.55 min, 34.41 min, 36.49 min, 57.43 min and 38.43 min respectively (Figure S2, available with the online version of this paper). As shown in Table 1, 2-propyl-1-pentanol; 3,5-di-tert-butyl-4-hydroxybenzaldehyde; 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione and dibutyl phthalate leached from both brands A and B. No documentation regarding deca-6,9-diene-2,8-dione and dibutyl phthalate leached from PE pipes, which were able to promote growth of E. coli. Growth rate of E. coli declined with the reduction of nutrients, which was consistent with the microbial four growth stages: the period of adjustment, the logarithmic phase, the stabilization period, and the decline phase. In addition, growth rate in brand B soaking solution was obviously higher than in brand A. Compared to brand A, brand B have another compounds of 2,4-di-tert-butylphenol and 2,6-di-tert-butylphenol, which represents that they are more easily absorbed by E. coli.

Table 1 | List of the most abundant organic compounds found in water which was in contact with two brands of PE pipe samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Organic compounds</th>
<th>Brand A</th>
<th>Brand B</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>2-propyl-1-pentanol</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>b</td>
<td>2, 4-di-tert-butylphenol</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>3, 5-di-tert-butyl-4-hydroxybenzaldehyde</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>d</td>
<td>2, 6-di-tert-butylphenol</td>
<td></td>
<td>√</td>
</tr>
<tr>
<td>e</td>
<td>7, 9-di-tert-butyl-1-oxaspiro(4, 5)deca-6,9-diene-2,8-dione</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>f</td>
<td>Dibutyl phthalate</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

Microbial experiments

To examine bacterial growth in the soaking solution of the PE pipes, E. coli was cultured and OD curves were determined. Soaking solution from brands A and B had a positive effect on the growth of E. coli, the growth rate reached a maximum in 16% and 7% for brands A and B after 5 to 8 h, respectively, and decreased afterwards (Figure 2(a)). The reason is that organic compounds of PE pipe leached as nutrients, which were able to promote growth of E. coli. Growth rate of E. coli declined with the reduction of nutrients, which was consistent with the microbial four growth stages: the period of adjustment, the logarithmic phase, the stabilization period, and the decline phase. In addition, growth rate in brand B soaking solution was obviously higher than in brand A. Compared to brand A, brand B have another compounds of 2,4-di-tert-butylphenol and 2,6-di-tert-butylphenol, which represents that they are more easily absorbed by E. coli.

The determination of the growth curve is based on the absorbance of the broth to determine the growth of E. coli. In this study, the plate count method was also used to determine the growth of living E. coli (Figure 2(b)). The colony count of the E. coli (from 0 to 24 h) was about 20 CFU/100 μL in the reference sample, but it has very large fluctuations in the soaking sample. The colony count of (b) was about 500 times more than that of (b). The colony count of (a), (b), (c) and (d) has been almost unchanged with the longer culture time. Colony count of (a'), (b'), (c') and (d') increased at first and then decreased with the culture time. Therefore, organic compounds from the PE pipe could be nutrients of microbial organisms and growth of microorganisms would be promoted at a certain extent. Microbiological growth in DWDS leads to the deterioration of water quality, which could cause health problems, so it is very important to control organic compounds leaching from PE pipes into drinking water.

Cytotoxicity in terms of MTT reduction

Toxicology and human clinical studies on some chemical substances leaching from polymer materials indicate that they have a variety of adverse effects on some target organs/systems, including liver, kidney, central nervous and immune system, etc. (Stern & Lagos 2008). The comprehensive toxic effect of leaching organic compounds on cells has not been understood clearly yet. Relative cell viability was larger than 100% under the dosage of 6 μL/well (Figure 3(a)), which suggested that low dosage of soaking solution would not inhibit the growth of C6 cells. Organic
compounds began to inhibit its growth when the dosage increased to 33 μL/well, and the inhibitory effect increased with increasing dosage. Over 30% C6 cells of brands A and B were inhibited at a dosage of 100 μL/well. The results provided us the possibility to assess drinking water pipe quality standards and health evaluation.

Cell culture morphology was checked by a phase contrast inverted microscopy at 200 magnifications. In the contrast group (Figure 3(b)), the cell structure was intact. However, the cell shapes appeared more and more round and transparency decreased, cellular morphologies became unhealthy (Figure 3(b)). This indicated that growth of C6 cells was affected significantly by leaching organic compounds from PE pipes.

**CONCLUSIONS**

In summary, residual chlorine, temperature and time have a positive effect on released organics, while pH plays an opposite role. The released amount of organics in tap water was greater than in soaking solution and was also greater than in ultrapure water. Most of the leaching organic compounds, such as aldehydes, alcohols, ketones, esters and phenols, are plasticizer and antioxidant. In this study, 2-propyl-1-pentanol has been found in PE pipe soaked water for the first time. Potential organics could promote the growth of *E. coli* as nutrients within limits. Organics exert an increasing inhibition on C6 cells with increasing concentration over a dose level of 33 μL/well. The above results could provide
the technical basis for updating and revising the drinking water pipe quality standards and health evaluation.

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