New method for urea analysis in surface and tap waters with LC-OCD-OND (liquid chromatography–organic carbon detection–organic nitrogen detection)

Stefan A. Huber, Andreas Balz and Michael Abert

ABSTRACT

A new method for urea detection in surface and tap waters using LC-OCD-OND was developed. It could be shown that urea, ammonia and nitrate could be separated chromatographically from each other and from NOM (natural organic matter). A direct quantification at its specific retention time is possible using custom-made detectors for nitrogen and for organic carbon. Further, urea can be detected indirectly as urea is transformed to ammonia after enzymatic hydrolysis with urease. After sufficient contact time for complete hydrolysis to ammonia (about 320 minutes) urea concentration can be calculated on the basis of the additional ammonia measured by LC-OCD-OND. The limit of detection of urea as mass was determined as 4 ppb for natural waters and 1 ppb for deionised waters.

Key words | natural organic matter (NOM), organic carbon detection (OCD), organic nitrogen detection (OND), size-exclusion chromatography (SEC), urea

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INTRODUCTION

Urea is a ubiquitous 'civilisatory' compound. About 80 to 90% of nitrogenous matter excreted by mammals via the kidney is urea. It is by far the most important synthetic fertiliser (Smil 2001). Urea is also an ingredient of many consumer products, including soaps, detergents and ointments. Urea is not considered harmful but, apparently, fermentation of juices containing urea (e.g. via degradation of arginine) may lead to the formation of ethyl carbamate which is considered carcinogenic at higher concentrations. The US Food and Drug Administration has released recommendations to minimise levels of ethyl carbamate in wine (Butzke & Bisson 1997), and techniques are described to remove urea from wine by immobilised urease (Andrich *et al*. 2010).

Urea is considered an important contaminant in the production of ultrapure water (UPW) for the semiconductor industry (Rydzewski 2002) because urea not only contributes to organic carbon, but it also releases ammonium, upon heating, which may negatively affect the lithographic process. Urea is non-ionic (not removed by ion exchange) and very low in molecular weight (poorly retained by reverse osmosis). No economic technique yet exists to remove traces of urea from water.

Urea plays an important role in the marine nitrogen cycle, and numerous papers on the analysis of urea in seawater are known and have been reviewed (Revilla *et al*. 2005). Two different approaches have been described. They are a direct approach, based on a colour reaction of urea with diacetylmonoxime, and an indirect approach based on the quantification of released ammonium after enzymatic reaction with urease. Both methods are well worked out and sensitive down to the ppb-range. Both methods should also be applicable to fresh water. A literature search with the keywords 'urea + tap (or drinking or potable) water' did not give a single match (20 October 2010).

The motivation to analyse natural water samples for urea came from the semiconductor industry. For chip manufacturing, ultrapure water (UPW) with TOC (total organic

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carbon) values in the low ppb range are used. In some UPWs a signal response for OCD (organic carbon detection) and OND (organic nitrogen detection) (not UVD [UV absorbance at 254 nm]) was found at the retention time of the permeation volume of the SEC (size-exclusion chromatography) column: around 73 min, thus in the low-molecular weight area. The compound is saturated (no response in UVD) and hydrophilic, otherwise the retention time would be longer. The compound was found to be non-ionic as changes in mobile phase composition had no impact on retention time. The response for OND in comparison to OCD gave a N/C molar ratio of 2:1. All this pointed to urea, and could be confirmed by comparison. In addition, a reactive control test was applied for safe identification. A small amount of urease was added to the sample, and the degradation of urea and formation of ammonium within a few hours was observed by re-measurement of the sample. An alternative test – heating an acidified sample to 70°C to hydrolyse urea – was in part successful but results are not conclusive to date. The aim of this paper is:

a) To introduce a new direct analytical method for the analysis of urea in water. This method is called LC-OCD-OND (liquid chromatography–organic carbon detection–organic nitrogen detection).
b) To show that urea is present in many tap waters, in particular if the raw water source is directly or indirectly (e.g. via bank filtration) connected to a surface water system.

MATERIAL AND METHODS

Description of the LC-OCD-OND system (Figure 1)

The liquid chromatographic system (also known as LC-DOC or SEC-OCD, Huber *et al.* 2011) uses size-exclusion chromatography to separate natural organic matter (NOM) from urea on a weak cation exchange polyacrylate resin with bead sizes of 30 μm and pore sizes of about 0.8 nm (TSK HW 50(S), 250 mm × 20 mm, Toso Haas, Japan). A mild phosphate buffer at pH 6.8 is used as a mobile phase (0.5 g KH_2PO_4 + 0.3 g $Na_2HPO_4 \times 2$ H_2O to 1 l, Fluka, # 30407 and # 30412). The mobile phase is made DOC-free on-line by UV-irradiation in an annular UV-reactor (80 cm length, 18 mm diameter with co-axial low-pressure mercury lamp of 7 mm diameter) (DOCOX, DOC-LABOR, Karlsruhe, Germany) placed upstream of the HPLC pump (flow rate 1.1 ml min^{-1}; S-100, Knauer, Berlin, Germany). For all analyses, 4,000 μl of non-buffered water sample was injected (autosampler MLE, Dresden, Germany). Samples were not pre-treated and not pre-filtered but samples were filtered after injection on-line over a 0.45 μm PES-filter (Sartorius, Germany, # 16537). The first detector after the chromatographic separation is a fixed-wavelength UV-detector (UVD, 254 nm, S-200, Knauer, Berlin, Germany) followed by an organic carbon detector (OCD, DOC-LABOR, Karlsruhe, Germany; Huber & Frimmel 1991). Before entering the OCD the measuring liquid is

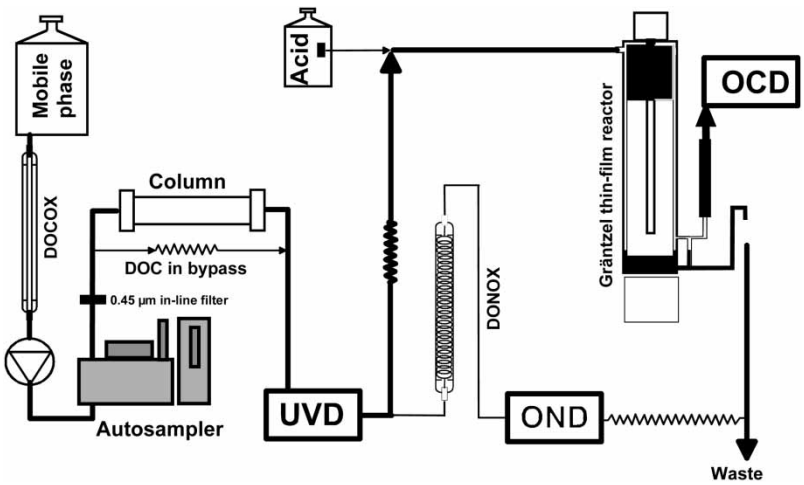

Figure 1 | Flow scheme of LC-OCD-OND.

acidified (gravity-driven; phosphoric acid 85% [Fluka #79620, 4 ml l^{-1}]). For nitrogen detection (OND), a T-piece behind the UVD allows a back-pressure-driven side stream (flow rate 0.1 ml min^{-1}) to enter a fused silica helical capillary of 4 m length and 1 mm inner diameter fused inside the electric discharge arc of a low-pressure mercury lamp (DONOX, 80 cm length, 18 mm diameter, DOC-LABOR, Karlsruhe, Germany). In this UV-reactor, organic carbon (OC) is converted to carbon dioxide while nitrogen bound to NOM, ammonium, nitrite or urea (or others) is converted to nitrate. Primary nitrate in the analyte remains unaffected. No oxidants are added as oxygen radicals are produced by cleavage of water at very short-wave ultraviolet irradiation at 185 nm (Huber & Frimmel 1991). Nitrate and nitrite are the only inorganic compounds that absorb strongly in the deep UV-range. This property is used to quantify the response as nitrate downstream of the DONOX reactor in a second UV-detector at 220 nm (K-2001, Knauer, Berlin, Germany). Nitrite should not be stable under these strongly oxidising conditions.

The OCD was calibrated with potassium hydrogen phthalate (Fluka 60359), and the OND with potassium nitrate (Fluka 31263). All fractions were quantified by area integration after peak fitting with customised software (ChromCALC, DOC-LABOR, Karlsruhe, Germany).

For the analysis of urea, the same system setting was used except for the mobile phase which was five times weaker in ionic strength. This opens the retention window for urea: nitrate elutes earlier (owing to stronger ionic repulsion of anions in the weak cation exchange column) and ammonium later (owing to stronger ionic attraction of cations in the weak cation exchange column). An earlier design of this set-up was used for the analysis of urea in ultrapure water at the ppt-level (Huber 2005).

The LC-OCD-OND system is normally used for the characterisation of humic and non-humic NOM. As an example, the chromatogram of a surface water (River Pfinz, Karlsruhe, Germany) is shown in Figure 2 for all three detectors. The bypass peak at 5 min is produced by a (pressure driven) capillary bypassing the column with 10% of liquid flow. This allows the calculation of matter 'lost' on the column.

Water samples and reagents

For assessing detection and determination limits of urea in natural water, sample tap water from Karlsruhe, Germany, was used. This is ground water taken at a depth of 60 m. The water had an average contact time in the aquifer of around 6 months. As reagents, urease from soy beans

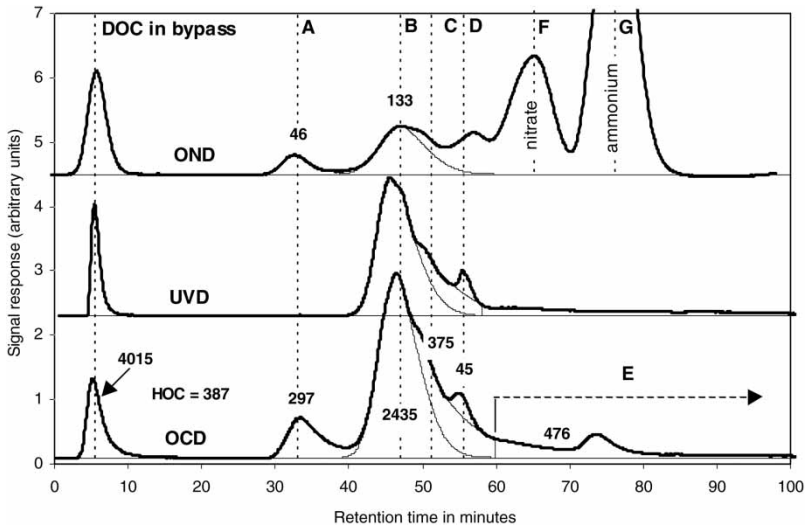

Figure 2 | Chromatogram of a surface water (river Pfinz, Karlsruhe, Germany) with LC-OCD-OND in the measuring mode for NOM. OCD, organic carbon; UVD, UV-absorbance 254 nm; OND, organic and inorganic bound nitrogen. Fraction designation: A = biopolymers; B = humic substances; C = building blocks; D = low molecular weight acids; E = low molecular weight neutrals; F, G = nitrate, ammonium (OND only). HOC = calculated difference between bypass and sum of chromatographic fractions. Values in OCD chromatogram are concentrations in ppb C; values in OND chromatogram are concentrations in ppb N.

(1.3 U mg^{-1}; Fluka # 94280) and urea (microselect; >95%; Fluka # 51456) were used.

RESULTS AND DISCUSSION

Identification of urea with LC-OCD-OND in deionised water

Figure 3 shows the most important findings for organic carbon detection. For simplification, results for UVD were omitted.

Chromatogram 'A' is for urea alone dissolved in deionised and DOC-free water (weighed-in concentration 750 ppb as mass). The OCD signal area corresponds to 155 ppb as mass C which gives a calculated value of 775 ppb for mass urea (mass/C-ratio: 5.0), or a recovery of 103%. The OND signal area corresponds to 315 ppb as mass N, which gives a calculated value of 734 ppb for mass urea (mass/N ratio: 2.14), or a recovery of 98%. On the molar basis the ratio is 1.90 which is slightly lower than the theoretical value of 2 owing to an excess recovery in OCD. Two conclusions can be drawn from these findings: (i) urea is recovered quantitatively from the column; and (ii) urea is quantitatively converted to carbon dioxide in the OCD and nitrate in the OND.

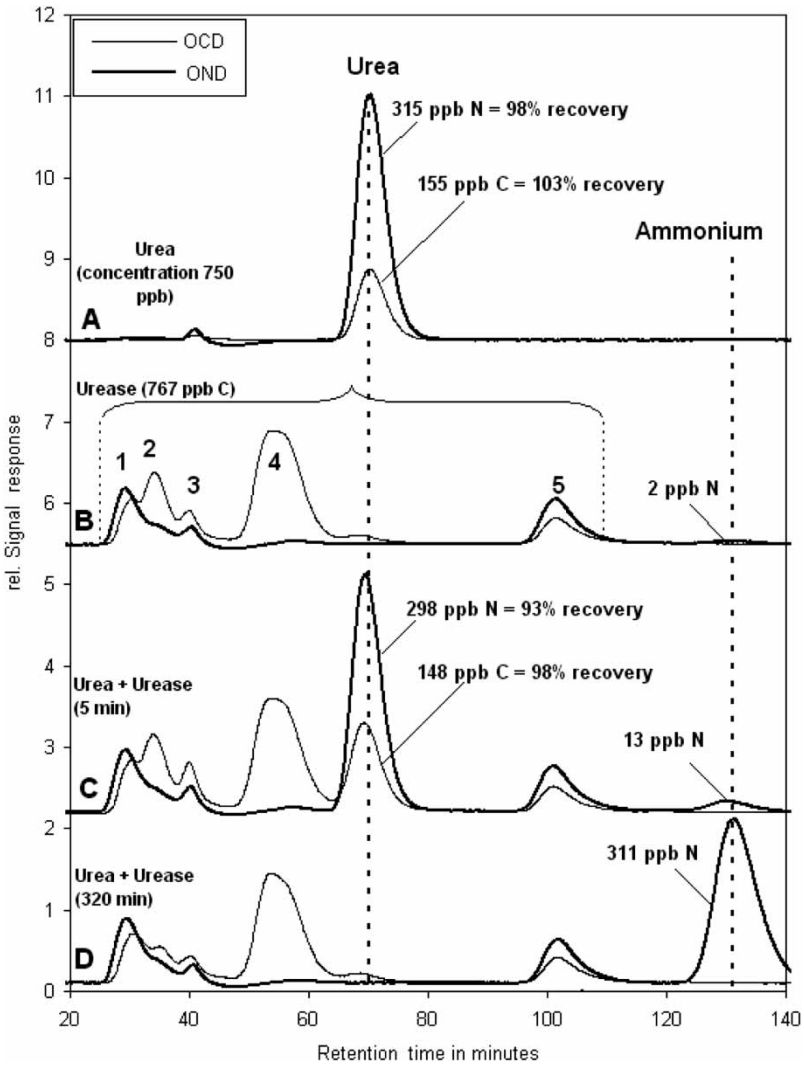

Figure 3 | Identification of urea with LC-OCD-OND: deionised water matrix.

Chromatogram 'B' (Figure 3) shows urease alone. As it was technically not possible to weigh in a defined amount of urease, an aliquot of a spatula tip was added. A separate DOC (dissolved organic carbon) analysis showed that about 80% of urease was recovered in the chromatogram. Surprisingly, urease did not elute as a single compound; five compounds in total are discernible which are chemically quite different. Compound 1 is high in molecular weight (>150 kDa by comparison with external weight calibration) and rich in N. Compound 2 has a nominal weight of around 1,000 Da (external weight calibration) and is low in N. Compound 3 elutes at the retention time of the 'missing buffer' in the sample which distorts the normal elution behaviour. Thus, compound 3 is an induced system peak. It is argued that compound 1 essentially elutes as a broad peak between 25 min and 50 min and is overlain by compound 2. Compound 4 is free of N and low in molecular weight. Finally, we find a slightly hydrophobic compound low in molecular weight and rich in N eluting at 105 min. We think that compounds 1 and 3 are urease, and compounds 2, 4 and 5 are impurities or degradation products. Ammonium was found with 2 ppb N.

Chromatogram 'C' (Figure 3) shows urea plus urease after about 5 min contact (reaction) time. Once the sample is injected, urea and urease are separated in the column with urea travelling at a much slower speed than urease. After 5 min contact time a partial degradation of urea and emergence of ammonium is already observable. The initial value of 315 ppb for urea-N is almost recovered (298 ppb urea-N + 13 ppb ammonium-N = 311 ppb).

After 320 min contact time (chromatogram 'D') all urea-N was converted to ammonium (311 ppb ammonium-N compared with initial 315 ppb urea-N). Also for OCD the urea peak has completely vanished, a still visible slight shoulder is related to urease (see chromatogram 'B' for comparison).

As we show, LC-OCD-OND offers two largely independent methods for quantification of urea. The first, direct method is quantification of the urea peak. The second, indirect approach is quantification of ammonium after enzymatic degradation. This approach follows in principle the indirect methods for urea analysis described in literature (review in Revilla *et al.* 2005).

Identification of urea with LC-OCD-OND in natural waters

In natural water samples the N/C ratio can often not be used for identification owing to co-eluting NOM compounds. If the ratio is 2 then it points to urea, if it is less then urea may be overlain by other organic matter. Irrespective of this, the urease degradation test should be a decisive criterion for identification. Figure 4 shows a natural surface water before and after spiking with urease (648 ppb as C). As in the previous test we find a slightly lower value for urea (−3 ppb N) and a slightly higher value for ammonium (+1 ppb N), after a few minutes of contact. A statistical test would be necessary to assess whether the slight changes are significant or not. Be it as it may, after 320 min an almost complete breakdown of urea (residual concentration 6 ppb N) was observed, while ammonium increased from 63 ppb N to 102 ppb N. The mass balance for N is complete, chromatograms from top to bottom: 45 ppb N + 63 ppb N = 108 ppb N, 42 ppb N + 64 ppb N = 106 ppb N, 6 ppb N + 102 ppb N = 108 ppb N. Traces of urea have remained while full destruction was observed in the model water test. Conditions were the same except that the matrix was different (deionised water versus natural water) and the concentration of urea was different (eight times lower in the real water experiment). Findings can easily be explained by slower degradation kinetics due to lower concentration of reactant. There is no need to postulate interference of urease with the natural water matrix.

Detection and determination limits

Figure 5 shows a graph of the calibration functions for urea and ammonium obtained with OND in the ppb range from 10 to 100 ppb using deionised water as matrix. The graph shows that calibration is linear and slopes are identical. As for both species N was converted to nitrate in the UV-oxidation lamp, the finding is as expected. It shows also that both species can be quantified down to the ppb level.

For assessment of detection and determination limits, LODs and LOQs (limit of detection, limit of quantification) were determined according to DIN 32 645 (1994) for n = 6 and 95% probability. Using the calibration curve method, concentrations of urea of 2, 4, 5, 6, 8 and 10 ppb,

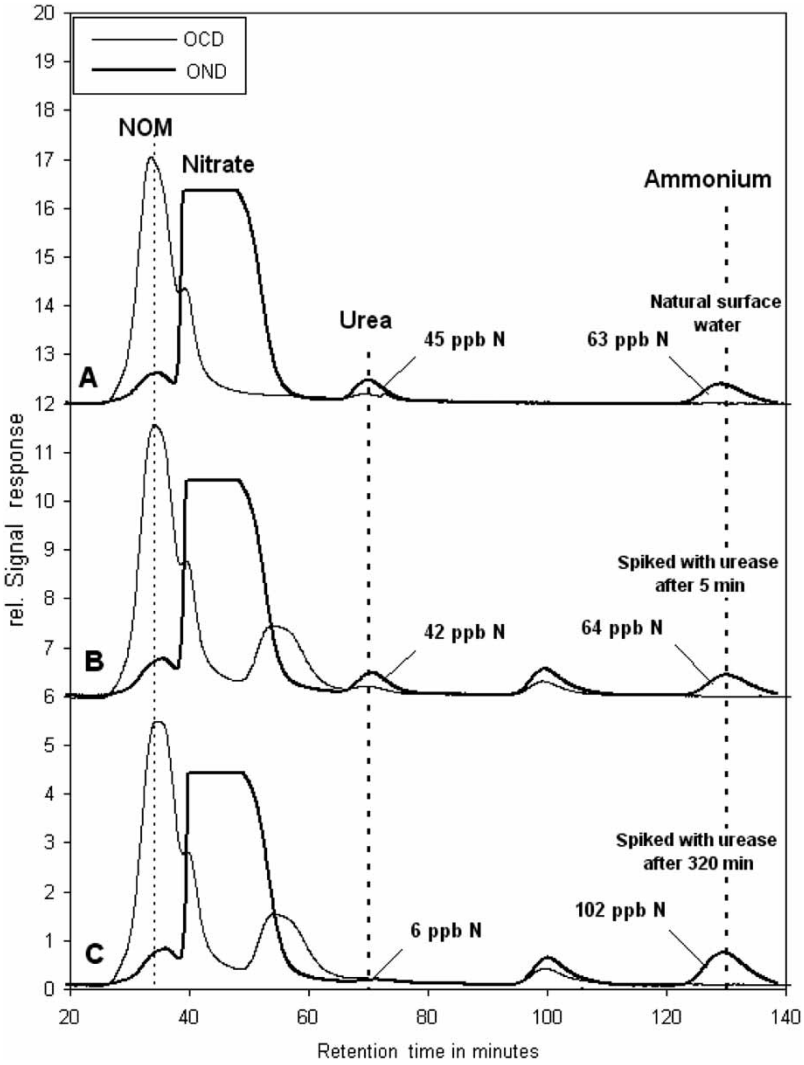

Figure 4 | Identification of urea with LC-OCD-OND: natural water matrix.

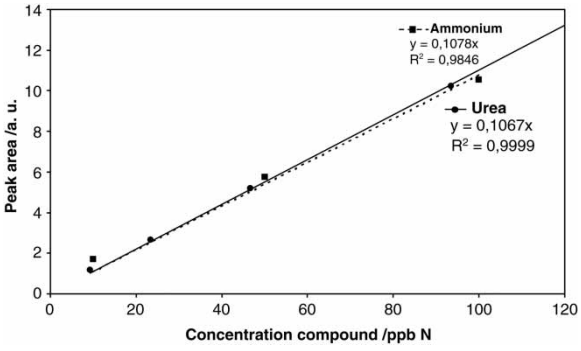

Figure 5 | Calibration functions for urea-N and ammonium-N at the 10 ppb to 100 ppb level.

respectively, were added either to deionised water (run 1) or to tap water (Karlsruhe, Germany) which is – according to our analyses – devoid of urea and can be used as blank water.

The LOD in deionised water was 1 ppb urea, while in tap water it was 2 ppb. The LOQ in deionised water was 2 ppb urea. In tap water it was 7 ppb.

Occurrence of urea in natural waters

It is too early to make general statements on the distribution of urea in natural waters. Only a few analyses have been carried out to date, but some broad assumptions can be made.

Diffuse sources would be run-off of urea from top soils spread with fertiliser or manure, or run-off from roads if urea is used as de-icing agent. Point sources would be primary to tertiary effluents. Thus it is likely that urea will be found in most surface water systems if these are influenced by human activity.

As an example, the occurrence of urea in a riverine system is shown in Figure 6 to illustrate high spatial variability in a river system fed from a single catchment area. Urea concentrations as urea-N (mass urea is urea-N $\times$ 2.33) vary from 21 ppb N to 488 ppb N. This shows that point sources play an important role in urea distribution. Figure 6 depicts another aspect which has not been raised before. Concentrations for urea and ammonium correlate quite well. While it is possible that both species originate from the same source, the degradation of urea to ammonium in the water bodies by ubiquitous urease is an aspect that has not yet been considered.

Occurrence of urea in tap waters

A selection of different tap waters was analysed to determine the concentrations of urea depending on the raw water resource and the respective water treatment scheme. It was assumed that its occurrence is related to the origin of the water. Should the tap water contain

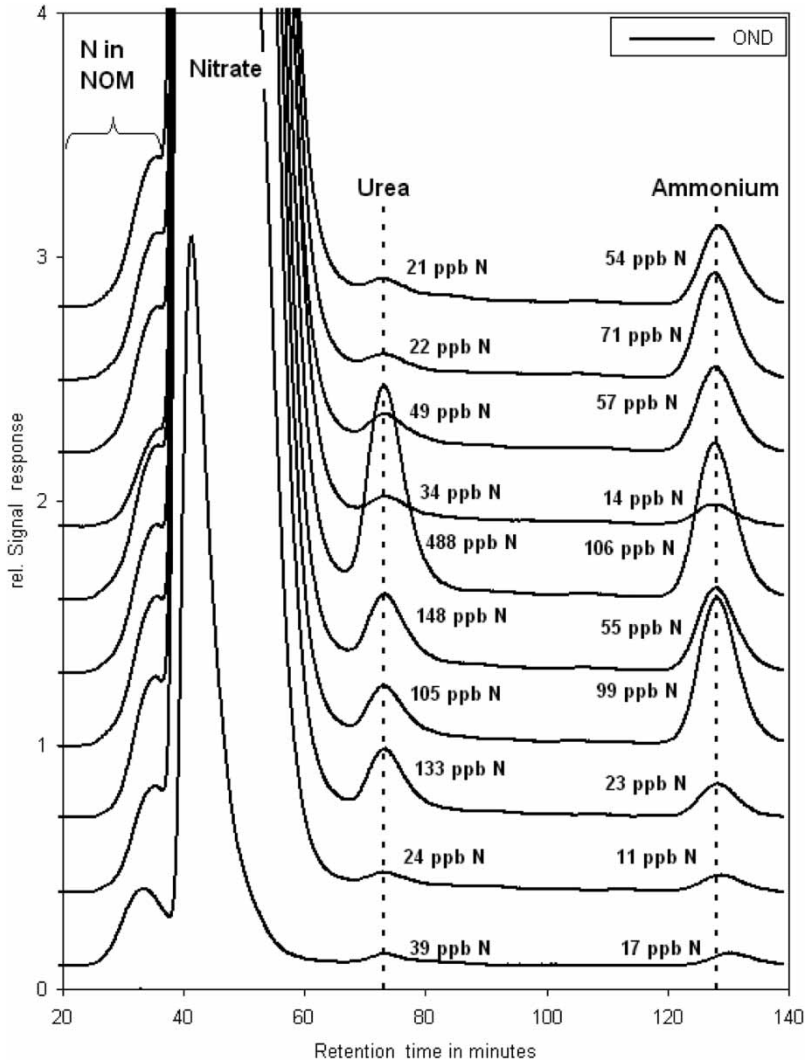

Figure 6 | Distribution of urea-N (urea mass = urea-N x 2.33) and ammonium-N at various points along a riverine system.

Table 1 | Presence of urea as mass in a selection of tap waters (MBR = Membrane bioreactor; RO = Reverse Osmosis)

Sample	Site	Source of Water	Urea as Mass
DW1	Worms/Germany	deep ground water	<1 ppb
DW2	Bretagne/France	shallow ground water	<1 ppb
DW3	Oblast Moscow/ Russia	shallow gound water	<1 ppb
DW4	North Germany	bank filtrate	22 ppb
DW5	not known	surface + ground water	51 ppb
DW6	Thuringia/ Germany	lake water	163 ppb
DW7	Far East/Asia	waste water MBR + RO	212 ppb

proportions of surface water, including river bank filtrate, the probability is higher that urea is present. For ground waters the likelihood of the presence of urea was considered to be very little since ground waters are in long-term contact with microbial communities living in the aquifer.

The assumptions could be confirmed (Table 1). Tap water 1 (DW 1) is from a deep well, urea is below the detection limit and also nitrate concentration is extremely low (<2 ppb urea-N). DW 2 and DW 3 are from shallow wells and there is also no urea found; DW 4 is from a bank filtrate; DW 5 is a blend of surface and ground water; DW 6 is largely from surface water; while DW 7 is wastewater/reclaimed water from a pilot plant.

SUMMARY

According to the literature search, urea in tap water seems not to be a focus in water research. With this article it is shown, probably for the first time, that urea is present in many natural waters and may also be present in tap waters. LC-OCD-OND was the technique used to analyse urea down to the ppb concentration level. Identification of urea with this technique is based on:

1. Retention time
2. N/C ratio (if applicable)
3. Absence of response in UVD
4. Degradation by addition of urease, and emergence of ammonium by addition of urease

It is speculated that urea could be an interesting marker compound for human impact. Provided that contribution of wild mammals to urea is negligibly low, then it can be said that urea in natural waters reflects input from human activities from effluents, from farmers spreading fertiliser or manure, or from authorities using urea as de-icing agent.

Future research should include the distribution of urea in both pristine aquatic systems and those subject to human influence, degradation rates in wastewater treatment plants, urease degradation kinetics and the behaviour of urea in different water treatment processes.

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