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► **To cite this version:**

Sylvie Rabouan, Antoine Dupuis, Axelle Cariot, Marion Albouy-Llaty, Bernard Legube, et al.. Metrology in Environment and Biology: The case of Nonylphenols in Environmental Health. 2012. hal-04286480

HAL Id: hal-04286480

<https://hal.science/hal-04286480v1>

Preprint submitted on 15 Nov 2023

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Metrology in Environment and Biology: The case of Nonylphenols in Environmental Health

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Abstract

Over recent decades, the development of environmental risks along with their repercussions on health has led to the appearance of a scientific research field of environmental health in which interdisciplinarity is intrinsic to the subject. This article on nonylphenols (NP) shows how exchanges and knowledge transfer involving chemists, biologists, pharmacists and physicians underscore the need to further the development of analytic methodologies in a context involving an ultimate objective likewise subject to constant development. As an example, when an objective consisting in assessment of NP presence in the environment so as to evaluate the quality of natural environments is transformed into an objective consisting in assessment of human exposure to the selfsame NP so as to evaluate risks to human health, it becomes indispensable to redefine the choice of substance of reference to be used. After having spelled out the difficulties encountered when selecting a substance of reference for the analysis of nonylphenols (multiplicity of isomers, variability in the composition of batches for the same CAS Registry Number), 353NP (CAS 186825-36-5) has been proposed as the substance of reference (high proportion in the industrial mixture and pronounced estrogenic power).

Highlights

> review of nonylphenols naming and standards generally applied in NP determinations > 353NP analysis in environmental and drinking waters > Argued choice of 353NP as the standard in studies for public health risk evaluation.

Keywords

nonylphenols, 353NP, standard, environment, health, water, exposure science

1. Introduction

High-level official recognition of the influence of the environment on public health was accorded in 1994 at the Helsinki conference organized by the European office of the World Health Organization. A charter on environment and health was drawn up by the members. It describes rights and obligations of the persons, governments and other actors involved ; it also formulates fundamental principles of general public interest and announces strategic elements and action priorities [1]. European Environment and Health Strategy integrates information on the state of the environment, the ecosystem and human health, in order to render the assessment of the overall environmental impact on human health more efficient. The main thrust of the strategy is to fill the knowledge gap on the link between environment and health, in a first phase focusing on a number of priority adverse health effects, including the endocrine disruptive effects [2]. Based on the human health data 42 substances were listed as category 1, including nonylphenol [3] which are continuously introduced into the aquatic environment by means of industrial, agricultural and municipal effluents. Nonylphenols are found throughout the environment, particularly in water resources [4]. NP are of major concern to environmental public health due to their high potential for human exposure and their demonstrated toxicity. In animals, nonylphenol induces reproductive and developmental toxicity involving feminization, a decrease in male fertility and juvenile survival, malformation during embryo development, adverse effects on organ structure and weight, a decrease in embryonic survival and insufficient body growth [4, 5].

From an environmental standpoint, the consequences of exposure to physical, chemical and biological aggressors are multiple and generally long-term, while the number of persons exposed is often exceedingly high. Moreover, the circumstances and duration of exposure, be they in natural, domestic or professional environments, are hard to quantify. As a result, decision-makers are often compelled to take measures in situations characterized for the most part by the pervasive uncertainty of scientists; the consequences of their decisions may involve high economic stakes and raise questions of social acceptableness going above and beyond their strictly sanitary effects. The frame of reference existing at an international level is aimed at organizing the elaboration of scientific knowledge and at bringing it to bear with regard to public decisions. In this respect, risk assessment (science-based) is clearly distinguished from risk management (policy-based) [6, 7].

Classically, a risk assessment approach is composed of four distinct steps: (i) hazard identification, (ii) definition of dose-response relationships, (iii) population exposure assessment, (iv) characterization of the sanitary risks. Step (iii) is the subject matter of a methodology known as exposure science, and its objective in real-life situations is to identify and characterize contact with toxic agents and their penetration in an organism. Unlike

toxicology, it is based upon actual field observations; for that reason alone, exposure assessment is of pronounced interest [8, 9]. Human biomonitoring or biosurveillance is one component of exposure science, which revolves around analysis of environmental pollutants, or their metabolites, in the biological environment of the persons involved [10-12].

As concerns NP, hazard identification has been established [13, 14] and the definition of dose-response relationships is known in the animal through toxicological studies [13-16]. As for assessment of population exposure to NP, exposure science shall perform analyses of the pollutant in the environment of the persons being studied and shall also carry out biomonitoring, which will necessitate an appropriate choice of not only the biological sample (blood, urine, fat, maternal milk...), but also the molecule or metabolite involved. More generally speaking, when choosing it will be necessary to ensure that the risk assessment approach maintains methodological continuity with regard to the contaminant in both natural and human biological environments, and thereby allows the data to contribute to the characterization of sanitary risks (Step iv) through epidemiological studies.

The key aim of this article is to focus the scientist's attention on the analytical chemistry and metrological issues related to nonylphenols. First, we will spell out the difficulties encountered when selecting a substance of reference for the analysis of nonylphenols, then propose 353NP (CAS 186825-36-5) as the substance of reference and finally discuss this choice, in a case where the objective of assessing NP presence in the environment so as to evaluate the quality of natural environments is transformed into the objective of evaluation of human exposure to the selfsame NP.

2. Generalities on nonylphenol analysis

2.1. The nonylphenols present in the environment [4, 14, 17]

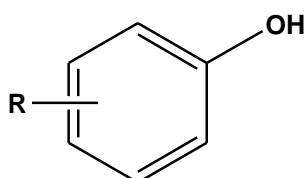
Nonylphenol ethoxylated derivatives (NPE) are one sub-set of a general group of compounds known under the name of alkylphenol ethoxylates. NPE are mass-produced chemical compounds. In Europe in 2002, as the result of the ethoxylation of nonylphenol with ethylene oxide, NP production neighbored 73500 tons. The most widely utilized alkylphenols are 4-tert-octylphenol (OP, CAS 140-66-9) and 4-nonylphenol (NP, CAS 84852-15-3). For more than 40 years, NPE have been used as detergents, emulsifiers, wetting agents and dispersing agents, and they are found in a large and wide-ranging number of fields, such as textile transformation, pulp and paper treatment, paints, resins for protective coating, recovery of gas and petrol, manufacture of steel, anti-parasite products and energy production. They are likewise found in a wide array of consumer products including cosmetics, cleaning products and paint. Lastly, NP can be used as an adjuvant (an antioxidant and a softener) in the manufacture of plastics such as epoxy resins and PVC. They are also NPE transformation products by means of microorganisms in the environment.

There exist no known natural sources of NP and NPE. The presence of these substances in the environment results solely from human activity. Release of NP and NPE in the environment may occur at different steps and stages of the products' life cycles, during primary NPE

production, manufacture of products containing NPE and NP, utilization of the products and their elimination in a wastewater treatment facility, a septic tank, town or factory landfill, or simply in the natural environment.

2.2. Naming NP

In general, the name NP specifies neither the location nor the isomer of the nonyl radical (R), of which the general formula is as follows:



Industrial NP is manufactured via Friedel–Crafts alkylation of phenol with technical nonene (“propylene trimer”: C₉-olefins with varying degrees of branching). Due to formation of carbocations during the acid-catalyzed process, the resulting NP mixture is highly complex, mostly containing para-substituted isomers with differently branched nonyl side chains [18, 19]. The approximate composition of the technical mixture is : 3-6% *o*-nonylphenol, 90-93% *p*-nonylphenol and 2-5% decylphenol [20]. Besides, approximately 50–80 nonylphenols, for 550 possible isomers, are found in biological and other environmentally relevant matrices [21].

There exist numerous CAS numbers for the Nonylphenol name, and the list in Table I is limited to the names pertaining to nonylphenols in para or predominantly para substitution – or to 4-NP –, since they are relevant to environmental issues. The table was drawn from the Sigma-Aldrich 2011 catalogue (sigma-aldrich.com), of the French *Institut National de Recherche et de Sécurité* (INRS)[13], from the Internet site of the Canadian government [22], and from the relevant ISO standards. In this paper, we are not dealing with CAS 11066-49-2 (predominantly ortho substitution of the nonyl chain) or CAS 139-84-4 (predominantly meta substitution of the nonyl chain). Up until 2009, the references CAS 186825-39-8 and CAS 186825-36-5 were not commercially available in any catalog (regardless of the supplier). The study described below was carried out with a locally synthesized substance of reference 353NP (CAS 186825-36-5) (cf. § 5. Material and methods).

Table I

This recapitulative list underlines how much it matters that the user of nonylphenol pay particular attention to the exact product he wishes to employ. On this score, standard ISO18857-2:2009 explicitly mentions the fact (in Table 1, page 2 of the document [23] that « CAS numbers 104-40-5 (4-nonylphenol, straight chain) and 25154-52-3 (nonylphenol, straight chain) have also been incorrectly used to denote the isomer mixture CAS 84852-15-3 ». The relevant standard indicates that « the commercially produced nonylphenols are predominantly 4-nonylphenol with a varied and undefined degree of branching in the alkyl groups. This mixture of isomers falls under the CAS number 84852-15-3». Issued the same

year, standard ISO24293:2009 nonetheless describes the substance of reference to be used with regard to the concentration of thirteen nonylphenol isomers corresponding to substitution isomers on the nonyl chain, which is itself in para substitution with regard to the phenol cycle and should be a product designated by the CAS number 25154-52-3 and described as a « technical mixture of isomers » (paragraph 4.8 page 3 of the document [24]). Given this apparent contradiction, we have created chromatographic imprints by GC-MS laboratory analysis of each one of the two products: CAS 25154-52-3 (batch N°50720, supplier Dr Ehrenstorfer, ref C15629000) and CAS 84852-15-3 (batch N° 1092230, supplier Fluka, ref 74430). In our analysis, CAS product 84852-15-3 (in para substitution) corresponded to around 75% of CAS product 25154-52-3. It would consequently seem to be the case that the technical mixture with CAS number 25154-52-3 is indeed a mixture of substitution isomers on the cycle and on the chain, and not simply (Table I) a mixture of molecules with linear radicals (as substitution isomers on the cycle). Conversely, CAS number 84852-15-3 presents a small percentage of substitution isomers on the cycle (other than in para substitution) as mentioned in the Sigma-Aldrich analysis reports: 90% minimum of ramified nonyl chain in position 4 ; in the impurities, substitution isomers on the cycle are once again reported, along with 0.2% of free phenol and to 3 to 4% of dinonylphenol. This observation corroborates the fact that industrial manufacture is not totally mastered as concerns the reproducibility of the batches, which are indeed complex and relatively variable according to the manufacturer and the batch number [25]. Moreover, in GC-MS laboratory analysis the presence of 4nNP (linear radical, CAS 104-40-5) was sought but not found in the two products, a finding signifying concentrations below 5% for CAS 25154-52-3 and CAS 84852-15-3.

It bears mentioning that in metrology in chemistry, the material (chemical substance) of reference must be of well-known and clearly defined composition, and we have just observed that for nonylphenols, (i) the products' degree of purity with regard to their description is variable, (ii) it can be found out by examining the analysis report, (iii) all the isomers of the mixture are not necessarily subjected to dosage during the control.

2.3. References of the nonylphenols used for control of water quality in accordance with the European directive, and in international scientific studies involving dosages in water and biological samples

The latest advances in monitoring chemical pollutants to assess water quality status in accordance with the Water Framework Directive and the attendant challenges were thoroughly reviewed in 2009 [26], and are listed in the Directive 2008/105/EC [27]. In the case of NP, the common position adopted by the European Parliament and the Council on 16 December 2008, on environmental quality standards in the field of water policy and in amending Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and 2000/60/EC, was to propose CAS 104-40-5 (4nNP) as Environmental Quality Standard (< 0.3µg/L when expressed as an annual average value, or < 2 µg/L when expressed as a maximum allowable concentration) with the mention that “Unless otherwise specified, it applies to the total concentration of all isomers”, even though the recommended standard method ISO 18857-2 [28] postulates CAS 84852-15-3 as material of reference, whereas ISO

24293 [24] postulates CAS 25154-52-3. In the case of France and as a response to the questions put forward by the *Agences de l'Eau* (French public establishments entrusted with implementation of the European directive) concerning the CAS n° to be used, a recommendation by in December 2010 the *Institut National de l'Environnement Industriel et des Risques* recommended for use as a standard a substance internally codified by the *Service d'Administration National des Données et Référentiels sur l'Eau* (SANDRE 6598) and containing nonylphenols CAS 84852-15-3, CAS 25154-52-3 and CAS 104-40-5 [29].

Table II and Table III list the different methods and substances used as references for the determination of NP in water and in complex matrices other than water, drawn from scientific literature published between 2002 and 2011. They show that the measurement benchmarks to be used may differ according to the studies in two significant respects:

- either designation of the nonylphenols with no indication of the CAS n°,
- or non-standardized expressions of results, that is to say variability with regard to the substance of reference (CAS n°) or to the means of calculation (with or without internal standard).

As for dosage in an aqueous environmental sample, Table II illustrates the wide variety of calculation methods and substances selected as material of reference, notwithstanding the harmonization texts established in 2006 with ISO standard 18857-1 (Water quality: Dosage of selected alkylphenols, part 1 published in 2006, updated in 2009) and then the ISO standard 24293 (Water quality: Determination of individual isomers of nonylphenol, published in 2009).

Considerations on the choice of substance of reference shall be presented later in this paper, particularly in the Discussion section.

Table II

Table III

3. Analysis of nonylphenols in the environment: An application case involving analysis of 353NP in water

3.1. Materials and methods

Commercial chemicals and reagents: Methanol, hexane, acetone, ethylacetate, dichloromethane of high analytical grade (Pestipur[®]) quality were purchased from SDS (Peypin, France). Sodium thiosulfate, internal standard 4*n*-NP-2,3,5,6-D4 were purchased from Sigma-Aldrich, Inc. Water was pre-treated by a Purelab Prima[®] and then purified by a Purelab classic (ELGA, Antony, France). Nitrogen alphasaz-1 was purchased from Air Liquide (Paris, France). 4-nonylphenol (diastereomer mixture of 4-(3,5-dimethylheptan-3-yl)phenol named 353NP) were custom-synthesized by @rtMolecule (Poitiers, France). Purity

was superior to 98%, and the ratio of diastereomer mixture synthesized in the present study was 353NP(E): 353NP(G) = 45:55 (according to the nomenclature proposed by Katase [25]).

Water samples: Inflow and effluent water (IW and EW) samples were collected from the 8 municipal drinking water treatment plants (DWTP) located in the French Poitou-Charentes region (n=16). Sampling was performed on three different days and samples were pooled before analysis. IW samples were directly collected at the river surface above DWTP and EW samples were collected at the outlet pipe of the treatment plant. EW samples were stored with the addition of a reducer (5 mg of sodium thiosulfate/250 mL) in order to stop chlorination of target compounds, all samples were kept frozen at -20°C until analysis.

Preparation of standard solutions: A 200 mg/L methanol stock solution of 353NP was stored at + 4°C. Extemporaneously, the initial stock solutions were diluted in methanol/water 50/50 (v/v) in order to obtain working standard solutions at 2, 4, 8, 20 and 40 µg/L (MeOH-standard). Internal standard solution was prepared in methanol/water 50/50 (v/v) at 20 µg/L from initial stock solution (200 mg/L).

Solid phase extraction: Prior to the extraction, 1.25 mL of methanol and 100 µL of the internal standard solution (50 µg/L) were added to an aliquot of 250 mL of water sample. Water samples were extracted by means of solid-phase extraction (SPE). The SPE procedure for clean-up and concentration of water samples was performed using a glass C18 upticlean® end-capped cartridge 200 mg (Interchim, Montluçon, France). Cartridges were conditioned with 4 mL of dichloromethane/hexane (50/50) and twice with 3mL of methanol/acetone/ethyl acetate (2/2/1) (v/v/v) and then equilibrated with 5mL of purified water. Water samples (250 mL) were passed through the wet cartridges, washed with 5mL of purified water and dried for 15 min. For all of these different steps, flow rate was set at 10 mL/min. Elution was performed with a mixture of hexane/dichloromethane 50/50 (v/v) (2x2mL) along with a mixture of methanol/acetone/ethyl acetate 2/2/1 (v/v/v) (2x2mL) at a flow rate of 2 mL/min. Extracts were evaporated at 40°C to dryness under a gentle nitrogen stream. Residues were dissolved in 100 µL of a purified water/methanol (50/50) solution. Finally, 25 µL of extract were injected into a LC/MS/MS apparatus.

LC-MS-MS analysis: Concentration of 353 NP was determined using an LC/MS/MS system consisting of an HPLC ultimate 3000 (DIONEX, Sunnyvale, USA), coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Forster City, CA, USA). HPLC column was a supercosil ABZ® 5 (3µm particle size, 150 mm x 4.6 mm) (Supelco, St Louis, USA). Mobile phase composition was methanol/eau 85/15 (v/v), using an isocratic solvent program. The MS/MS was equipped with an atmospheric pressure chemical ionization (APCI) interface, operating in negative ionization mode. Quantitative analysis was carried out in the multiple reaction monitoring (MRM) mode, using two specific combinations of a precursor-product ion transition for each compound. Retention times and precursor product transitions along with their corresponding collision energies are shown in Table IV. MS/MS detector conditions were set as follows : ion source gas 1 50 psig; probe temperature 550°C; curtain gas 20 psig; collision gas 9 psig, entrance potential -5.0 V, nebulizer current -1.

Table IV

Method validation: 353 NP contaminations may arise from laboratory accessories, reagent, SPE procedure, and apparatus. In order to avoid such contamination, only pre-treated glassware (500°C, 5 h), Teflon seals and high-quality solvent were used throughout the study. Two kinds of blanks have been performed. The first blank involved purified water with sodium thiosulfate addition as the sample loading step on which the SPE procedure was carried out; the second blank was obtained by SPE procedure without the sample loading step. Linearity of the chromatographic response was assessed on three different days using standard curves including 5 calibration points ranging from 2 to 40 ng/L. Recovery (R) was determined by analysis of n river water samples (exempt from target compounds) spiked at 2 different concentrations (20 ng/L and 40 ng/L) with 353NP (n=10) and 4nNPD4 (n=7). The recovery value includes matrix effect and losses during SPE. The limit of detection (mLOD) and the limit of quantification (mLOQ) of the method were calculated, respectively, as three and ten times the signal-to-noise (S/N) ratio in blanks, corrected with the recovery. Intra-day and inter-day coefficients of variation were calculated for 20 ng/L. Sample concentrations were determined using the corresponding MeOH-standard curve calibration and corrected by the recoveries ($C_{\text{water}} = C_{\text{inj}}/10R$, with C_{inj} as concentration in the injected solution and R as recovery of the compound). Intra-day coefficient of variation was obtained with five replicates of quality control sample at 20 ng/L. Inter-day coefficient of variation was obtained from experiments performed on three separate days with two replicates of quality control sample at the same concentration. Accuracy was assessed by measuring the ratio between calculated (C_{water}) and theoretical values.

3.2. Results

Method validation: 353NP was detected in blank extracts but at a level lower than the limit of quantification. Regardless of whether purified water was loaded prior to SPE procedure, the results were similar. Moreover, no trace of 353NP was detected following direct injection of solvents. A small degree of contamination appeared as the extraction technique was being applied, and it was taken into account as we calculated the results. Recoveries for 353NP and 4nNPD4 were respectively 49% (RSD 19%) and 35% (RSD 58%). Calibration curves of 353NP provided adequate linearity as shown by correlation coefficients greater than 0.99. The method provided mLOD and mLOQ values of 1.4 ng/L and 4.1 ng/L respectively for 353NP. Intra-day and inter-day variation obtained from quality controls of the 20 ng/L of 353NP were respectively 5.6% (n=5) and 14.9% (n=6), while accuracies were 98.3% and 89.2% respectively in intra-day or inter-day analyses.

Water sample analysis: Appropriate Internal Quality Controls were considered so as to evaluate the validity of the optimized analytical procedure and to check that no outliers occurred during routine analysis sequences. Signal specificity was systematically checked by comparison of each retention time and fragmentation ratio with the corresponding standard. Calibration curves were likewise regularly checked for linearity ($r^2 \geq 0.99$). Moreover, quantification of quality control (20ng/mL) was regularly performed during DWTP water sample analysis (bias $\leq 11.0\%$, RSD $\leq 17.0\%$). 353NP was detected in concentrations ranging from 13.5 to 124.9 ng/L in the IW and from <mLOD to 59.4 ng/L in EW (Figure 1).

Figure 1

4. Discussion

Analytical chemistry is a discipline whose key objective does not lie in the mere application of methods producing a numerical figure in exchange for an experimental sample [30]. Quite on the contrary, it plays an instrumental role in decision-making with regard to problems as wide-ranging and vital as medical diagnosis, quality control for industrial products, protection of the environment, and environmental epidemiology. After all, risk characterization as defined by the National Research Council (1983) [7] is continually enhanced by studies emanating from research in three areas where measurement matters: on the field, in toxicology, and in epidemiology. By basing its presentations on analytical chemistry methodology in the form of a triad bringing together (a) « the problem to be resolved », (b) « how the problem is framed » and (c) « the chemical information to be obtained » [30], the issues in environmental health pertaining to NP may be understood as follows : (a) consists in learning whether or not there are endocrine disruption effects to be observed in humans provoked by the NP present in the environment, (b) to consider that 353NP is the NP isomer liable to be largely responsible for endocrine disruption, because it is largely present in the environment, (c) to perform 353NP analysis in environmental water and biomonitoring.

Since NP in the environment involve more than 100 substitution isomers [21], exhaustive analysis of the latter is hardly realistic. And when the final objective consists in studying the endocrine disruption effects that may or may not be attributed to these molecules, the first priority is to decide on the molecule to be searched in the environment and a biological sample. The choice of the right molecule is contingent on the model, which is tantamount to the way the problem is framed. The model is a tool designed to detect the NP isomer bearing the brunt of responsibility for endocrine disruption effects. Researchers have got to define this NP isomer, a hormonally active agent otherwise known as an endocrine disruptor, which predominates in technical NP mixtures and is found in the environment and in biological samples. The arguments in favor of the 353NP molecule (CAS 186825-36-5) will be elaborated in the next paragraphs, in which we will respectively discuss the chromatographic separation and MS detection of NP, and the consequences of the modified objectives of NP analysis in the framework of environmental health on the NP analysis benchmark reference. Lastly, the final selection will be explained, along with the analytical procedure.

Chromatographic separation and MS detection are discussed: Due to the similarities of the chemical and physical properties of the many NP isomers, complete separation and identification of individual isomers remains quite difficult, if not altogether impossible. Routine analysis using gas chromatography with apolar capillary column yields separation of 13 p-NP isomers, which are constituted by more than 90% of the 4-nonylphenol isomers that are detectable in technical products and in environmental samples in general [24, 31]. One may note that no single 4nNP molecule appears in these technical mixtures (concentration below the detection limit). Much recent research has been focused on the application of highly

sophisticated coupling systems to the isomer-specific determinations of nonylphenol. For example, the coupling of two-dimensional gas chromatography and mass spectrometry (GCxGC/MS) has led to the identification of 80 to 110 different isomers in technical nonylphenol from different manufacturers [21, 32, 33]. It should also be noted that there exist 211 possible constitutional isomers of p-nonylphenol out of a total of 550 possible compounds taking into consideration the chiral C-atoms [18, 21]. The composition of isomers in the proposed reference varies according to the batch numbers, and their fragmentation fails to yield the same fragments [19], it becomes downright impossible to quantify with regard to the technical mixture, which consequently cannot function as a reference.

In the overall context of the Water Framework Directive, the reliability (quality control/assurance) and comparability of the analytical data gathered in a given environment are of paramount importance as they lead to enhanced demonstration of the ensuing measurements' correspondence to the established references [34], as occurred in 2009 for the ISO 18857-2 protocol, with the participation of 14 laboratories from 4 different countries in Europe and Canada [35]. The first approach (ISO protocol 18857-1:2006) adopted by the research community and the international measurement organizations is to proceed by GC/MS with regard to the sum of the isomer peak areas: particular attention must be paid to substances that co-elute with NP and yield the same ion(s) because the co-elution can interfere in the determination and exert substantial influence on the final result. ISO protocol 18857-2:2009 (with CAS 84852-15-3 as the standard) is aimed at ensuring that interfering peaks are excluded from the sum of the areas, and limits inclusion to the peaks from the sample attributable to the multicomponent analyte [28]. The second approach by GC/MS is proposed in ISO protocol 24293:2009 (with CAS 25154-52-3 as the standard), and it consists in quantifying specific isomers identified by their retention time along with two selected diagnostic ions; the specificity of the signal used has imperatively got to be verified.

The two choices are both satisfactory in terms of the objective of analysis proper to the ISO standards, which is to quantify NP in the environment [35]. But when the objective of a study is to assess sanitary risk by means of biomonitoring (assays in biological samples) and environmental analysis (water) in order to study their correlation, these two methods of analysis are no longer suitable, for reasons to be explained later.

The final objective of the study has been modified: Using the preceding benchmarks, the sum of the signals recorded in the samples for the sought-after analytes is compared to the sum of the signals emanating from the mixture of reference and expressed in terms of concentration with regard to the substance of reference. If the proportions of the different isomers are the same in the samples of reference as in the unknown quantities, this mode of calculation engenders practically no evaluation error in determination of the NP content (in the form of a mixture). In actuality, all relevant findings lead us to believe that the relative proportions of the different isomers to be found in unknown biological samples are bound to differ from those to be found in a reference mixture, which corresponds to the raw material utilized in industry ... and to the source of biological sample contamination! Three separate arguments lead to the same conclusion:

- a. The relative proportions of the different NP isomers vary from one manufactured batch to another, which means that the substance of reference is not the same when the batch number has been modified (between two different or identical manufacturers)
- b. How each of these different isomers evolves in the environment cannot be known. All the literature tells us is that (i) nonylphenol would certainly appear to be the main product of degradation of nonylphenol ethoxylates, (ii) does not undergo further transformation and (iii) is strongly adsorbed onto the sludge solids [4]. As for the pattern of relative of NP isomer concentrations, it has been found to vary from environmental sample to the next [36, 37]. The proportion of isomers in the environmental sample subjected to analysis will inevitably differ from the proportion in the substance of reference.
- c. The same goes for the proportions of the isomers in the biological samples subjected to analysis. No data in conjunction with bioconcentration for isomers such as “partition coefficient” (K_{ow}) are presently available. The rare published studies on the subject indicate nothing other than $\log K_{ow}$ for the technical NP mixtures [38, 39], and in a study on 353NP bioconcentration, it is pointed out that “a correlation between the metabolism and the K_{ow} seems to be unlikely, and that two mechanisms triggering the bioconcentration (partitioning between two phases and biotransformation)” can be distinguished from each other and “described both mechanisms with a simple weight-dependent bioconcentration model [40]». Another study mentions considerable algal bioaccumulation, but the material & methods section does not indicate the CAS n° used in NP [41].

To sum up, if the scientific community wishes to pose as an objective the comparison of studies seeking to draw a connection between environmental NP content and human impregnation levels, it is necessary to use the same substance of reference for environmental analyses and biomonitoring. In its absence, comparative studies are pointless, and information with regard to exposure science will remain limited.

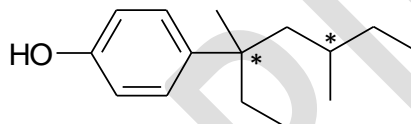
The final selection is explained : It is consequently necessary to seek out a reference NP, present in the environment *per se* and in the biological environment, composed of a substance defined by its chemical formula and of known purity, in order to express the results in terms of a precisely defined molecule. In Table I, CAS 84852-15-3, CAS 25154-58-3 and CAS 90481-04-2 are isomer mixtures and cannot be selected, nor can CAS 26543-97-5, CAS 87247-00-5 and CAS 104-40-5, which are indeed a single substance, but have not been employed industrially in the production of ethoxylated NP, and are consequently of no relevance to studies of the environment or of environmental health. Moreover, the 4*n*NP isomer (linear radical, CAS n° 104-40-5) is not detectable (<5%) in the technical NP mixtures used for industrial purposes, and research in surface water and drinking water ([42],[43],[44]) shows that the compound is either absent or minimally present (< quantification limit), as is the case with concentrations in urine [45].

The substance of reference liable to be selected will be one of the branched isomers of the technical mixture used by industry (CAS n° 84852-15-3). Which one? Along with analytical development aimed at quantifying nonylphenols in the technical mixture, numerous

researchers have studied the estrogenic power of the main isomers contained in industrial mixtures [25, 46-50], specifically in 353NP [47, 51, 52]. Since the isomer 3E2NP (CAS 186825-39-8, Table I) is not one of the isomers studied by the authors consulted while this article was being written, it will not be taken into account. On the contrary, if the 353NP para-nonylphenol molecule (CAS 186825-36-5) has been selected as a reference in this work of ours, it is for the following reasons : (i) it represents from 12 to 20 % of the isomer mixture CAS 84852-15-3, and through the *MVLN* test, the 353NP molecule significantly contributes to the estrogenic effect with regard to CAS 84852-15-3; (ii) the 3E22NP para-nonylphenol molecule, which presents the most pronounced estrogenic power through the *YES* test, represents only 4 to 6% of the CAS 84852-15-3 mixture.

Finally, 353NP has been shown to be present in living organisms using a radiolabeled nonylphenol isomer to investigate the bioconcentration in *Daphnia magna* [40].

The 353NP nomenclature corresponds to 4-(1-ethyl-1,3-dimethyl-pentyl)-phenol. It contains two asymmetrical carbons and is presented in the form of a diastereomeric mixture of 353NP(E) and 353NP(G) (according to the nomenclature proposed by Katase [25]).



Using the nomenclature proposed by Guenther, this is molecule 111, as described in « Electronic Supplementary Material » [21].

Our analytical procedure is detailed: In the work presented in this paper, we attempted to detect the presence of 353NP in environmental water and at the entrance to a domestic water treatment plant (DWTP), and we then analyzed the water coming out of the plant. Our goal was to propose a protocol for analysis and to evaluate the presence or absence of this molecule in the environment. High-purity solvents and glass - instead of plastics – were used and specific purification procedures were performed, but they did not avoid 353NP contaminations, which were probably due to leaching effects during solid phase extraction. Other studies have also reported NP contamination in blank [53]. In the present study, 353NP contaminations did not exceed the limit of quantification, but still, special attention needs to be paid when proceeding. During MS parameter optimization, negative ionization mode produced higher signal intensity than positive ionization mode and a much better signal-to-noise ratio (S/N). APCI (atmospheric pressure chemical ionization) and ESI (electrospray) interfaces have been compared using both methanol solution of compound and spiked river water. ESI interface produced higher sensibility using methanol solution. Due to a significant matrix effect, the best results in spiked water were obtained using APCI interface, which was consequently chosen for use in this study.

In this work, 4*n*NP-2,3,5,6-D4 was initially envisioned for use as the internal standard, based on the recommendation of the 4*n*NP(ring-¹³C₆) in the ISO24293:2009(E), and because the presence of 4*n*NP has been reported in environmental samples [42, 44], the unlabeled 4*n*NP cannot be employed as internal standard. As our method was drawn up, however, it became

obvious that the physicochemical properties of 4nNP-2,3,5,6-D4 differ too pronouncedly from those of 353NP to be an acceptable choice. Not only is retention time appreciably longer, (6.05 min instead of 4.45 min for 353NP), but it presents a weak and excessively variable yield of extraction (35% with RSD of 58%). Others authors [54] have also shown that structure and shape of this molecule with a linear alkyl chain differ markedly from the isomers in the technical mixture, especially in the response factor by GC/MS, so they have proposed a 4-NP isomer with a secondary alkyl side chain (4-(2,6-dimethylhept-3-yl)phenol) to serve as internal standard. In the same way, ISO18857-2:2009(E) has recommended the 4-(3,6-dimethyl-3-heptyl)phenol(ring-¹³C₆) as internal standard. The results described herein consequently employ external calibration as a means of calculation, but use of an isotope labeled 353NP (deuterium or carbon-13) is recommended in order to be able to calculate by means of internal calibration. In experimental conditions, the 353NP isomer was characterized by its retention time (4.45 min, diastereomers E+G co-eluted) and the m/z fragmentation 219-133 and 219-147. In our water sample analysis results, 353NP presence is evaluated in terms of inflow and effluence of WDTP from surface water. 353NP was found in all surface water samples analyzed in this study at a level ranging from 13.5 ng/L to 124.9 ng/L. Similar or different results are reported in the literature, but these comparisons should be put forward with caution, given the complications we have mentioned about the NP standard to be used [53, 55-58]. Concentration decreased in most of the effluent water samples (Figure 1, Nr 1-2-4-6-7) from 2.2% to 100%, with a median of 100%. Authors from different countries reported similar results with overall elimination ranging from 73 to 100% [56, 58, 59]. On the other hand, we found more 353NP in some EW samples (than in corresponding IW), probably because our EW and IW withdrawals were carried out at the same time in the water treatment plant, without taking into account (water) flow time in the plant and the (unknown) fluctuations in 353NP concentrations in the IW, but it is also possible that some 353NP were released during drinking-water production and transport [60].

As a general rule, NP quantification may be carried out by 353NP analysis. Up until now, however, the methods used in NP analysis have failed to yield adequately distinct results, and it would consequently be preferable to select a single, clearly identified isomer.

5. Conclusion

The analytical data gathered in an environmental perspective constitute the foundations of the European water quality assessment system as described in the Water Framework Directive. In this context, reliability (quality control/assurance) and comparability of measurements are of paramount importance as they lead to enhanced demonstrations of the ensuing measurements' correspondence to the established references. In this article, we have detailed the difficulties encountered in selection of a substance of reference in the case of nonylphenol determination (multiplicity of the isomers, variability in the composition of batches for the same CAS number). What is more, the references to nonylphenols used in today's environmental analyses (CAS 84852-15-3 and CAS 25154-52-3, respectively in conjunction with standards

ISO18857-2:2009 and ISO24293:2009) are not transposable in assays of this micro-pollutant in biological samples.

Classically, a risk assessment approach involves four basic steps or stages, whose the exposure assessment which is meant to assess NP population exposure, through analyses of the pollutant in the environment of the persons studied and its biomonitoring. Here, the choice of the molecule to be analyzed at this stage has been shown to be the most pertinent with regard to 353NP (high proportion in the industrial mixture and pronounced estrogenic power). As a general rule, given that this choice should help to ensure that the risk assessment approach will be part and parcel of a methodological continuum, it is necessary to carry out follow-up not only in human biological samples, but also in water; the data gathered will consequently contribute to the characterization of sanitary risks (step iv) by means of epidemiological studies.

In this work, the concentration at the entrance and outlet of the water treatment plant showed that in evaluation of 353NP presence in terms of inflow and effluent of WDP from surface water, it was found in all surface water samples analyzed at a level ranging from 13.5 ng/L to 124.9 ng/L, and also remained in some effluents (drinking water). Moreover, Preuss *et al.* have shown 353NP bioconcentration, using U-ring-C¹⁴-labeled 353NP, in daphnies [40].

To conclude, while 353NP assays do not allow for quantification of all the NP, evidence of its presence in the environment and during biomonitoring can allow for estimation of NP exposure. That's why the 353NP molecule (CAS 186825-36-5) can be recommended for the purposes of studies of not only the quality of environmental and drinking water, but also of epidemiology studies, in which correlations are sought between presence of the contaminant in the environment and its presence in biological samples. As a result, the sizable number of environmental data gathered during analysis campaigns could be used in an epidemiological context and thereby enhance assessment of the public health risk with regard to NP from an environmental health standpoint.

Acknowledgements

The authors wish to thank Pascale Pierre-Eugène for her technical assistance, the Ianesco laboratory (Régis Brunet and Marie-Jeanne Gourmaud, Poitiers, France) for their contribution to the LC/MS/MS analyses, the @rtMolecule laboratory (Fabien Zunino and Omar Karam) for their know-how in organic synthesis, and all of them for having so generously shared their work experience. They are also highly grateful to the AFSSET (study EST-2007-70/LEGUBE) for its financial contribution to the project. Finally, they wish to thank Jeffrey Arsham for having provided the English translation.

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Table Captions

Table I: List of the names and CAS numbers of the different para-nonylphenols, drawn from the Sigma-Aldrich catalogs 2011, from the INRS, from the Canadian government Internet site, and from the ISO standards.

Table II: The different nonylphenols of reference used in the literature (examples) for assays in water (environment and drinking water).

Table III: The different nonylphenols of reference used in the literature (examples), for assays in complex matrices other than water.

Table IV: MS/MS parameters

PRE-PRINT

Figure caption

Figure 1 : ³⁵³NP concentration in water (inflow and effluent of eight drinking water treatment plants)

PRE-PRINT

Table I

CAS Nr	Usual name	Description
104-40-5	4-nonylphenol 4- <i>n</i> -nonylphenol <i>p</i> -nonylphenol	Linear radical
84852-15-3	branched 4-nonylphenol	In para substitution, but as an isomer mixture with ramified radical (without linear radical) <i>for Sigma-Aldrich 90% minimum in position 4 with mention of substitution isomers on the cycle.</i>
25154-52-3	nonylphenol	Isomer mixture according to <i>INRS FT249 and ISO 18857-2 :2009 mixture of substitution isomers on the cycle of the linear nonyl radical.</i> <i>according to ISO 24293:2009 isomer mixture in para substitution for the ramified nonyl radical.</i>
90481-04-2	branched nonylphenol	Ramified radical (in isomer mixture), and mixture of substitution isomers on the cycle
26543-97-5	<i>p</i> -isononylphenol	Isononyl radical in para substitution
87247-00-5	<i>p</i> -tripropylphenol	Tripropylene radical in para substitution
186825-39-8	3E2-nonylphenol	4-(2-ethyl-1-methylhexyl)phenol <i>Available in catalog since 2009</i>
186825-36-5	353-nonylphenol	4-(1-ethyl-1,3-dimethylpentyl)phenol <i>Available in catalog since 2009</i>

Table II

Bibliography (year)	Technique	Nonylphenol of reference as mentioned in the publication	Supplier	Method with internal standard
^a (2002)	GC/MS	CAS 25154-52-3	not mentioned	absent
^b (2005)	Not indicated	CAS 25152-52-3 CAS 104-40-5	not relevant	
^c (2005)	GC/MS	4- nonylphenol	Sigma-Aldrich	bisphenol A-d16
^d (2005)	HPLC/(ESI)MS	Technical grade 4-nonylphenol	Aldrich	4-heptylphenol (aldrich)
^e (2006)				
^f (2006)	GC/MS AFNOR – Europe ISO 18857-1	CAS 84852-15-3	Not relevant	4- <i>n</i> -nonylphenol (ring- ¹³ C ₆) ou 4- <i>n</i> -nonylphenol (CAS 104-40-5) if absent from the sample to be dosed
^g (2006)	GC/MS	Technical 4-nonylphenol (NP)	Riedel de Haen	4- <i>n</i> -nonylphenol (CAS 104-40-5; Riedel de Haen)
^h (2007)	HPLC (Fluo)	4-nonylphenol	Lancaster	4- <i>n</i> -nonylphenol (CAS 104-40-5 ; Riedel de Haen)
ⁱ (2007)	HPLC/ (ESI)MS/MS	4-nonylphenol CAS 84852-15-3	Aldrich	4- <i>n</i> -nonylphenol d8 (Dr Ehrenstorfer)
^j (2008)	HPLC/ (ESI)MS/MS	4- <i>n</i> -nonylphénol	CIL Cluzeaux	absent
^k (2008)	Laboratory (included 7) intercomparison study GC/MS without derivatization or GC/MS with derivatization or LC/MS/MS or HPLC (Fluo)	4-nonylphenol CAS 84852-15-3	Not mentioned	4- <i>n</i> -nonylphenol d8 or 4- <i>n</i> -nonylphenol d6 or 4-bromophenol or atrazine d5 or without internal standard

^l [45](2009)	Interlaboratory trial (included 14) according to ISO 18857-2 GC/MS with derivatization	NP	Sasol	4-(3,6-dimethyl-3-heptyl)phenol (ring-13C6) Abbrev.: 363 NP-13C6
^m [38] (2009)	GC/MS ISO 18857-2	CAS 84852-15-3	not relevant	4-(3,6-dimethyl-3-heptyl)phenol (ring-13C6) Abbrev.: 363 NP-13C6
ⁿ [35] (2009)	GC/MS ISO 24293-1	CAS 25154-52-3	not relevant	4- <i>n</i> -nonylphenol (ring- ¹³ C ₆)
^o [63] (2010)	HPLC/ (ESI)MS/MS	Nonylphenol CAS 84852-15-3	not mentioned	4- <i>n</i> -nonylphenol d8 (Dr Ehrenstorfer)
^p (2010)	VALLME/HPLC (Fluo) (VALLME=Vortex Assisted Liquid-Liquid MicroExtraction)	Nonylphenol	Riedel de Haen pestanal®	absent
^q [53] (2011)	LLLME/HPLC (Fluo) (LLLME=Liquid-Liquid-Liquid MicroExtraction)	4- <i>n</i> -nonylphenol	Alfa Aesar Johnson Matthey	absent
^r [54] (2011)	LC/MS/MS	4- <i>n</i> -nonylphenol	Supelco	absent
^s (2011)	GC/MS	NP	Fluka	<i>n</i> NP

^a Kolpin *et al.* Environ Sci Technol. 36 (2002) 1202. ^b French Ministry of Ecology, Water Framework Directive, Chemical and Ecological Status of Water in France ^c Wang *et al* Anal Bioanal Chem. 383 (2005) 857. ^d Cespedes *et al* Chemosphere. 61 (2005) 1710. ^e Cespedes *et al* Anal Bioanal Chem. 385 (2006) 992. ^f ISO18857-1:2006 ^g Voutsas, P. Hartmann, C. Schaffner, W. Giger *et al.* Environ Sci Pollut Res Int. 13 (2006) 333. ^h Ghanem *et al.* Chemosphere. 69 (2007) 1368. ⁱ Loos *et al.* Chemosphere. 66 (2007) 690. ^j Stavrakakis *et al.* Environ Technol. 29 (2008) 279. ^k Loos *et al.* Trends in Analytical Chemistry. 27 (2008) 89. ^l Stottmeister *et al.* Analytical Chemistry. 81 (2009) 6765. ^m ISO18857-2:2009(E) ⁿ ISO24293:2009(E) ^o Loos *et al.* Water Res. 44 (2010) 2325. ^p Yiantzi *et al.* Talanta. 80 (2010) 2057. ^q Lin *et al.* Journal of Separation Science. 34 (2011) 428. ^r Jardim *et al.* Separation and Purification Technology. *in press* (2011) ^s Amiridou *et al.* J Hazard Mater. 185 (2011) 1, 281.

Table III

Bibliography (year)	Matrix	Technique	Nonylphenol of reference mentioned in the publication	Supplier	Method with internal standard/Quality control (QC)
^a (2005)	urine	GC/MS	4- <i>n</i> -NP (linear)	absent	Isotope (name not indicated)/QC
^b (2007)	eggs and milk	LC/MS/MS	4- <i>n</i> -NP technical purity NP	Tokyo Kasei Kogyo	absent
^c (2008)	breast milk	GC/MS	nonylphenol	ChemService	Dodecylbenzene
^d (2009)	soil	TD/GC/MS	4-nonylphenol (technical mix)	Aldrich	absent
^e (2009)	breast milk and milk	GC/MS	4-nonylphenols technical grade	Aldrich	cumylphenol
^f (2010)					
^g (2011)	powdered milk	HPLC/MS/MS	NP	Aldrich	absent

^a Calafat *et al.* Environ Health Perspect. 113 (2005) 391. ^b Shao *et al.* J Chromatogr B. 850 (2007) 412. ^c Ademollo *et al.* Environ Int. 34 (2008) 984. ^d Llorca-Porcel *et al.* Journal of Chromatography A. 1216 (2009) 5955. ^e Lin *et al.* Food Chemistry. 114 (2009) 753. ^f Chen *et al.* Food and Chemical Toxicology. 48 (2010) 1939. ^g Ferrer *et al.* Food Chemistry. 126 (2011) 360.

Table IV : MS/MS parameters

Compound	Retention time (min)	MRM(m/z)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	Dwell time (ms)
353NP (E+G)	4.45	219-133	-70	-35	-20	40
		219-147	-70	-26	-11	40
4nNPD4	6.05	223-110	-80	-40	-30	40

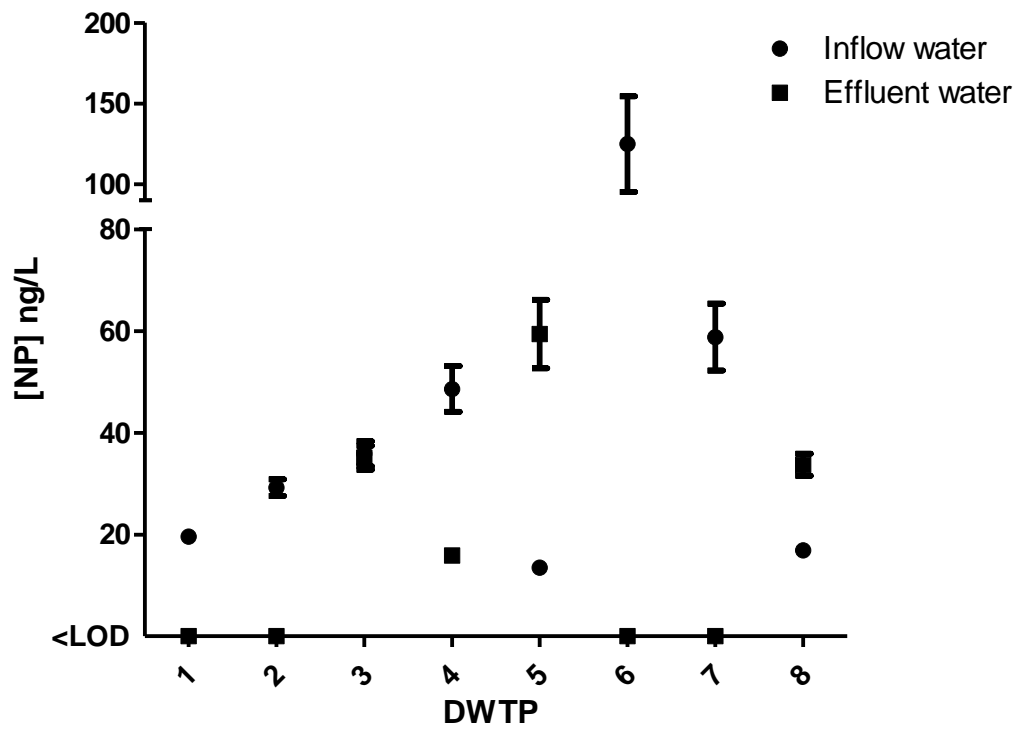


Figure 1