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Understanding Toxicity and Formation of Chlorinated Products of 1,3-Diphenylguanidine (DPG) in Water

Using real-time cell analysis and triple-quadrupole MS

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Abstract

This application note presents the evaluation of 1,3-diphenylguanidine (DPG) and five of its chlorinated derivatives' effects on cell heath and bioenergetics using the Agilent xCELLigence RTCA eSight and Agilent Seahorse XF96 extracellular flux analyzer. 1-(2,4-dichlorophenyl)-3-phenylguanidine (CC15) and 1-(4-chlorophenyl)-3-(2,4-dichlorophenyl)guanidine (CC11) were the least and most cytotoxic compounds, respectively, except for DPG, which was the most cytotoxic. RTCA results were similar to traditional alamarBlue cell viability assay results. The Seahorse XF96 analyzer results indicated that, except for 1,3-bis-(2-chlorophenyl)guanidine (CC04), all of the DPG compounds tested had adverse effects on cell bioenergetics. While the compounds' limited effects on mitochondrial basal respiration and ATP production might be short lived, the significant effect on proton leakage suggested irreversible mitochondrial damage.

After characterizing the compounds' real-time effects on live cells, the formation of compounds produced by reaction of DPG with chlorine and monochloramine under conditions found in water treatment and distribution networks was evaluated. Detection and quantification of target DPG and DPG derivatives used an Agilent 6495C Triple Quadrupole LC/MS system operated in multiple-reaction-monitoring (MRM) mode. Lower chlorination doses up to 40 µM favored the formation of di-halogenated DPG products. Tri-halogenated and tetra-halogenated DPG products were not detected. The target chlorinated products were not observed during the chloramination experiments. The high concentrations of CC04 and CC15 produced during simulated chlorination are a concern, particularly for chlorinated waters that come in direct contact with pipes or other components capable of leaching DPG. Though CC04 was only formed at low concentrations, its potential toxicity is a concern due to its effects on cell viability and cell bioenergetics.

The combination of the *in vitro* assays evaluated and the characterization of by-product formation potential by triple quadrupole LC/MS offers a workflow applicable to environmental toxicology screening early in product development to reduce hazardous chemical release into the environment.
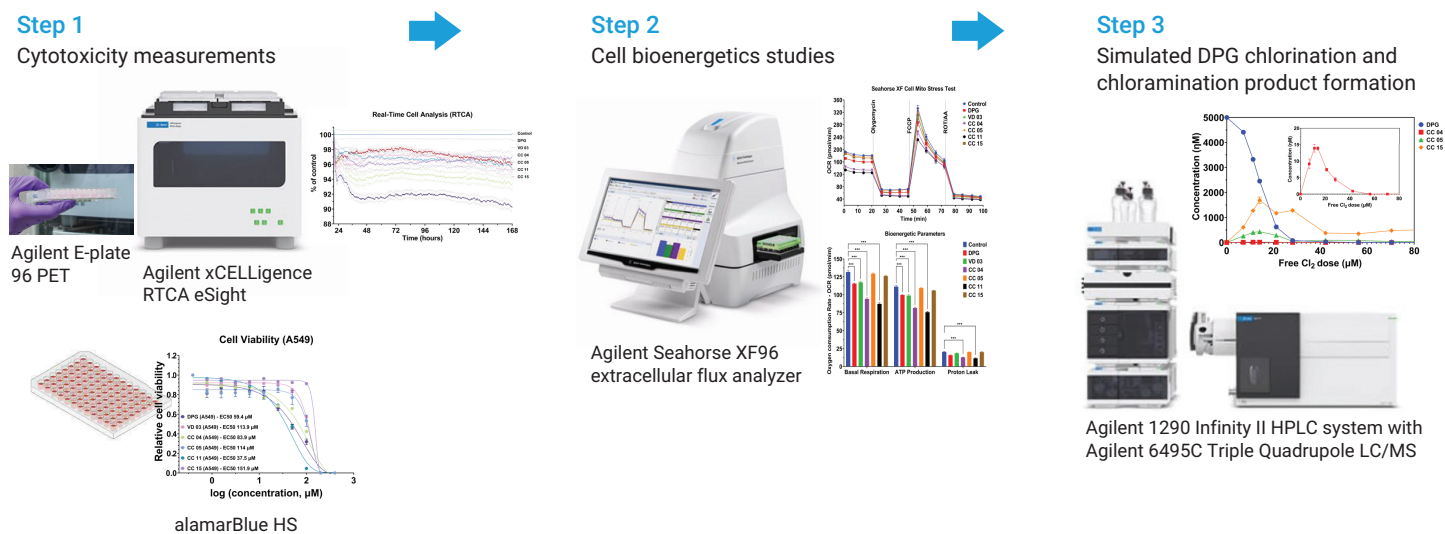
Introduction

1,3-diphenylguanidine (DPG), one of the chemicals used in the manufacture of rubber and other polymers[1], is a major component released from tires during their wear and aging.[2] DPG is found around the world at low ng/L to mg/L concentrations. For example, analysis of water from the Rhine river and its tributaries revealed a median DPG concentration of 41 ng/L and a maximum of 140 ng/L.[3] Other studies of European surface waters identified DPG concentrations ranging from 5 to 100 ng/L.[4,5] Surface water testing in Canada found DPG in all samples, with peak concentration of 0.52 µg/L.[6] In the US, up to 540 ng/L was detected in Washington State.[7] In Japan, up to 467 ng/L have been detected in surface waters.[8] Studies have also shown that DPG migrates from high-density polyethylene (HDPE) pipes, which have been used in municipal water applications for many years, resulting in concentrations in water ranging from 0.12 to 0.74 mg/L.[9]

Though DPG is a known allergen[10], its effects on environmental health are not fully understood. In water treatment, known disinfection by-products (DBPs) explain only a small fraction of the observed genotoxic effects.[11] Several studies have demonstrated an increase in genotoxicity of chlorine-disinfected waters using *in vitro* testing[12], *in vivo* evidence,[13] and epidemiological studies.[14]

This application note describes the evaluation of DPG and five of its chlorinated derivatives' effects on cell heath and bioenergetics based on *in vitro* assays using the Agilent xCELLigence RTCA eSight and Agilent Seahorse XF96 extracellular flux analyzer. The compounds were selected based on previous literature, mainly Sieira *et al.*, 2020[15], preliminary tests using high resolution mass spectrometry (HRMS) analysis, and laboratory ability to synthesize standards. RTCA analyses were compared to traditional alamarBlue cell viability assays.

After evaluating the compounds' real-time effects on cells, experiments were carried out to characterize the formation of compounds produced by reaction of DPG with chlorine and monochloramine under conditions mimicking those found in water treatment and water distribution networks. Detection and quantification of target DPG and DPG derivatives produced in the simulated chlorination experiments used an Agilent 6495C Triple Quadrupole LC/MS system operated in multiple-reaction-monitoring (MRM) mode. The three main parts of the study are shown in Figure 1. The *in vitro* cytotoxicity and cell bioenergetics assays evaluated, with triple quadrupole LC/MS characterization of simulated by-product formation, offers a workflow applicable to environmental toxicology screening early in product development with the goal of reducing the number of hazardous chemicals released into the environment. Mauricius Marques dos Santos *et al.*[16] developed the methods described in this application note.

Step 1
Cytotoxicity measurements

Agilent E-plate 96 PET

Agilent xCELLigence RTCA eSight

alamarBlue HS

Step 2
Cell bioenergetics studies

Agilent Seahorse XF96 extracellular flux analyzer

Step 3
Simulated DPG chlorination and chloramination product formation

Agilent 1290 Infinity II HPLC system with Agilent 6495C Triple Quadrupole LC/MS

Figure 1. Three part workflow used to explore the toxicity and formation of chlorinated products of 1,3-diphenylguanidine (DPG) in water.

Experimental

Chlorinated DPG products synthesis

1,3-bis-(4-chlorophenyl)guanidine (CC04), 1,3-bis-(2-chlorophenyl)guanidine (CC05), 1-(4-chlorophenyl)-3-(2,4-dichlorophenyl)guanidine (CC11), 1-(2,4-dichlorophenyl)-3-phenylguanidine (CC15), and 1,3-bis(2,4-dichlorophenyl)guanidine (VD03) were synthetized chemically and purified as described by Mauricius Marques dos Santos *et al.*[16] The five compounds were selected based on previous studies, preliminary tests using HRMS, and ability to synthesize standards.

Cell culture

Cell viability after exposure to DPG and its chlorinated products was studied using a lung carcinoma epithelial cell (A549) line. Cells were maintained at 37 °C and 5% CO_2 atmosphere in DMEM cell culture media supplemented with 10% fetal bovine serum (FBS). The media was exchanged twice a week and the cells were sub-cultured before reaching confluence (80 to 90%). Cells were counted by trypan blue exclusion using an automated cell counter.

Cytotoxicity by alamarBlue HS cell viability analysis

AlamarBlue assays have been frequently used in environmental studies to determine cell viability. For the alamarBlue HS cell viability experiments performed in this study, 20,000 cells in 100 µL of DMEM phenol red-free media supplemented with 2% FBS were seeded into each well of a 96-well microplate and then incubated in an incubator with 5% CO_2 at 37 °C for 16 hours. The test compounds were dissolved in DMSO to a stock solution of 20 mM. Compounds were then added to cells at concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 µM, with six replicates per concentration (n = 6). The concentration of DMSO was held at 0.05%. DMSO was also used as a control. After 24 hours of incubation at 37 °C and 5% CO_2, 10 µL of alamarBlue reagent was added to each well and the plate was incubated for another 2 hours at 37 °C and 5% CO_2. After final incubation, fluorescence signal (Ex = 560 nm; Em = 590 nm) was measured using a microplate reader.

Cytotoxicity by real-time cell analysis (RTCA)

Cellular impedance measurements were performed using the xCELLigence RTCA eSight. The xCELLigence RTCA eSight enables comprehensive insight into cell health, behavior, function, and processes using live, simultaneous, real-time biosensor impedance-based and image-based measurements. 10,000 cells in 100 µL of DMEM phenol red-free media supplemented with 2% FBS were seeded into each well of a 96-well microplate (Agilent E-plate 96 PET). The E-plate 96 PET contains a set of gold microelectrodes fused to the bottom surface of each well that noninvasively quantify cell behavior. After incubation overnight for 16 hours, the DPG test compounds were added at 5 µM in concentration (n = 6 replicates). Though multiple concentrations were used in the alamarBlue assays, for the remaining bioassays only the highest non-cytotoxic concentrations of 5 µM were applied. Experiments were conducted at 37 °C and 5% CO_2. The impedance was recorded every 15 minutes for 600 cycles (6.25 days).

Cell bioenergetics and mitochondrial stress tests

Cell bioenergetics experiments were performed using the Seahorse XF96 extracellular flux analyzer. The analyzer is designed to measure oxygen consumption rate (OCR)—a key indicator of mitochondrial respiration, glycolysis, and ATP production rate—in live cells in a 96-well plate format. The analyzer performs compound addition and mixing, label-free analytical detection, and automatic measurement of OCR in real time. 20,000 cells per well were seeded into the assay plates with 100 µL of culture medium. The cells were incubated at 5% CO_2 for 16 hours at 37 °C, and then treated with different concentrations of the compounds under study (n = 6 replicates) for an extra 24 hours. After exchanging the media to DMEM extracellular flux media, the impact of the test compounds on the cells' OCR was measured with the addition of compounds that disrupt mitochondrial function. Oligomycin (an ATP synthase inhibitor), FCCP (a protein gradient uncoupler), and rotenone/antimycin A (an electron transport chain disruptor) were added to final concentrations of 1 µM at 20 minutes, 1 µM at 50 minutes, and 0.5 µM at 70 minutes, respectively and the OCR (pmol O_2/min) was measured over 100 minutes. A standard DAPI assay of cell number was used to normalize the OCR measurements obtained.

DPG chlorination and chloramination: simulated product formation

Subsequent experiments were carried out to explore formation of products produced by reaction of DPG with chlorine and monochloramine under conditions mimicking those found in water treatment and distribution networks. Water chlorination experiments were carried out in 20 mL of 5 mM phosphate buffer (pH 7.0), spiked with 5 µM of DPG from a stock solution of 10 mM in ultrapure water. Samples were treated with free chlorine concentrations ranging from 7 to 80 µM for 10 minutes (n = 3 replicates). Residual chlorine was measured using the DPD colorimetric method. The reaction was quenched with 125% molar excess of ascorbic acid. Chloramination experiments were carried out in a

40 mM monochloramine solution and the concentrations of preformed NH_2Cl and $NHCl_2$ were measured using UV-Vis spectrometry. The monochloramine solutions were prepared as described by Mauricius Marques dos Santos *et al.*[16]

Total chlorine and NH_2Cl residuals were measured by the DPD and Indophenol methods. Additional kinetics experiments with 5 µM of DPG and 70 µM of oxidant (HOCl or NH_2Cl) at 30 seconds, and 1, 2, 5, 10, 20, 30, 45 and 60 minutes of contact time were also conducted. After reduction with ascorbic acid, 2 mL of each sample was transferred into an amber LC/MS vial with a PTFE/Silicone/PTFE cap for analysis by LC/MS/MS. Experiments were at room temperature (25 ±2 °C), and samples were kept at 4 °C and analyzed within 24 hours.

LC/MS/MS analysis of DPG and chlorinated products formed during simulations

Quantitative analysis of DPG and its chlorinated products formed during the simulations were performed using an Agilent 1290 Infinity II HPLC system coupled to an Agilent 6495C Triple Quadrupole LC/MS system operated in MRM mode. The LC instrument parameters are shown in Table 1. Separation was carried out on an Agilent InfinityLab Poroshell 120 EC-C1 column (2.1 × 100 mm, 1.9 µm) with an Agilent InfinityLab Poroshell 120 EC-C18 UHPLC guard (2.1 × 5 mm, 1.9 µm). To reduce carry over, 20 µL of aqueous solutions were injected via an Agilent multiwash injector.

Table 1. LC instrument parameters.

Parameter	Value
LC	Agilent 1290 Infinity II HPLC system with multiwash injector (p/n G7167B #112)
UHPLC Guard	InfinityLab Poroshell 120 EC-C18, 2.1 × 5 mm, 1.9 µm (p/n 821725-940)
Column	InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 1.9 µm (p/n 695675-902)
Column Temperature	40 °C
Injection Volume	20 µL
Binary Pump Flow	0.4 mL/min
Solvent A	Water + 0.1% formic acid
Solvent B	Acetonitrile + 0.1% formic acid
LC Gradient	Time (min) A (%) B (%) 0 95 5 1 95 5 8 40 60 9 0 100 10 0 100
Post Analysis Time	3 minutes

The MS instrument method parameters, including MRM transitions, are shown in Table 2. The source parameters were selected using the Agilent MassHunter Source Optimizer. The optimum precursor and fragment ions were selected using Agilent MassHunter Optimizer. The MassHunter Optimizer is method development software that automatically optimizes the data acquisition parameters for MRM experiments carried out on an Agilent Triple Quadrupole LC/MS system. The Optimizer selects the best precursor ions, optimizes the fragmentor voltage for each precursor ion, selects the best product ions, and optimizes the collision energy of each transition for a user-specified set of compounds, providing significant time savings compared to manual optimization procedures. The 6495C Triple Quadrupole LC/MS system was controlled by Agilent MassHunter Acquisition Software. Data analysis used Agilent MassHunter Quantitative Analysis Software (version 10.1). The external calibration curves for quantitation were prepared using concentrations ranging from 2.5 to 50,000 ng/L (50 ng/mL).

Table 2. Triple quadrupole LC/MS instrument and MRM method parameters.

Parameter	Value
Mass Spectrometer	Agilent 6495C Triple Quadrupole LC/MS system
Gas Temperature	150 °C
Gas Flow	15 L/min
Nebulizer	40 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12 L/min
Capillary	3,500 V
VCharging	1,500
Pos High Pressure RF	70 V
Pos Low Pressure RF	40 V
Fragmentor	166 V
Collision Cell Accelerator	5 V

MRM Method Parameters				
Compound	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	Retention Time (min)
DPG	212.12	119.1	24	3.875
	(212.12)	(77.1)	48	
CC04	280.04	153	24	5.404
	(280.04)	(111)	50	
CC05	280.04	153	24	4.552
	(280.04)	(126)	36	
CC11	314	186.9	24	5.767
	(314)	(153)	28	
CC15	280.04	119.1	24	5.123
	(280.04)	(77.1)	48	
VD03	347.97	187	28	5.944

Results and discussion

## DPG and chlorination product cytotoxicity by alamarBlue HS cell viability analysis

The estimated half maximal effective concentration (EC_{50}) values for each compound were determined and compared. CC15 was the least cytotoxic with an EC_{50} value of 151.9 μM. Compared to the cytotoxicity of DPG, only CC11 produced lower EC_{50} values, indicating that these chlorinated products are more toxic than DPG. The cytotoxicity curves for cell line A549 are shown in Figure 2.
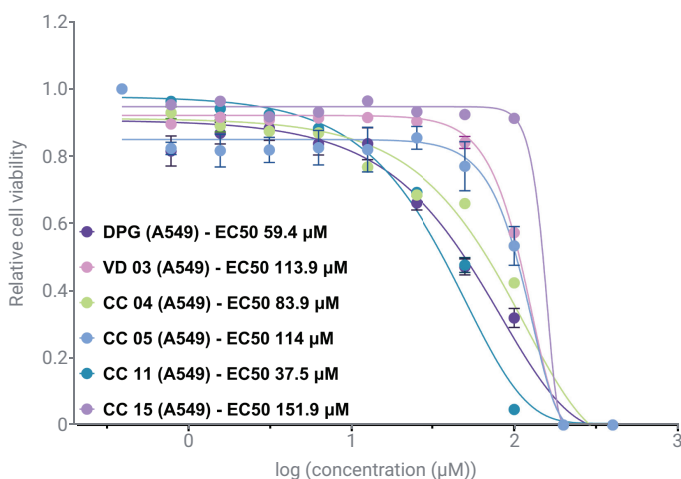
Figure 2. EC_{50} plot obtained from alamarBlue HS cell viability analysis. The compounds were added to A549 cells at 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 μM with n = 6 replicates.

DPG and chlorination product cytotoxicity by RTCA

In the initial 24 hours of exposure to DPG and with the chlorination products at 5 μM concentration, real-time cellular impedance measurements obtained from the xCELLigence RTCA eSight produced results similar to the alamarBlue HS cell viability analysis. Shown in Figure 3, at 24 hours, CC11, CC04, and DPG had the greatest impact on cell viability. However, after prolonged exposure at 168 hours, all of the chlorinated products tested were more toxic than DPG. The order of toxicity from lowest to highest was CC11 > CC04 > VD03 > CC05 > CC15 > DPG (Figure 2).
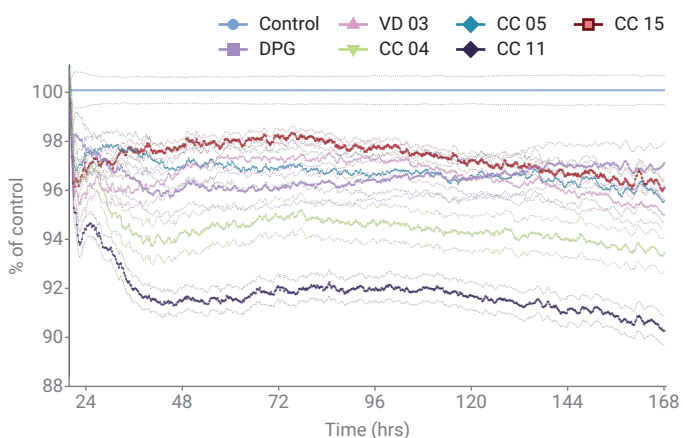
Figure 3. Results of RTCA cell toxicity studies are presented as % of control (DMSO at 0.05%).

Studies have demonstrated that impedance-based cytotoxicity assay results such as those produced by the xCELLigence RTCA eSight correlate well (R^2 >0.9) with traditional end-point cytotoxicity assays.[17] Despite the low number of compounds tested here with n = 6 replicates, the Pearson's correlation between the traditional endpoint AlamarBlue cytotoxicity assay and RTCA at 24 hours of exposure correlated well (R^2 = 0.847, p = 0.033). Although impedance-based cytotoxicity assays often have a higher initial cost, the automated, label-free, and real-time nature of RTCA assays simplifies the experimental workflow and examination of toxicity effects over various time windows with noninvasive and continuous monitoring. These benefits are in comparison to conventional cytotoxicity assays, which only provide single-time-point data. With the ability to run up to three 96-well plates, the xCELLigence RTCA eSight also allows for higher throughput.
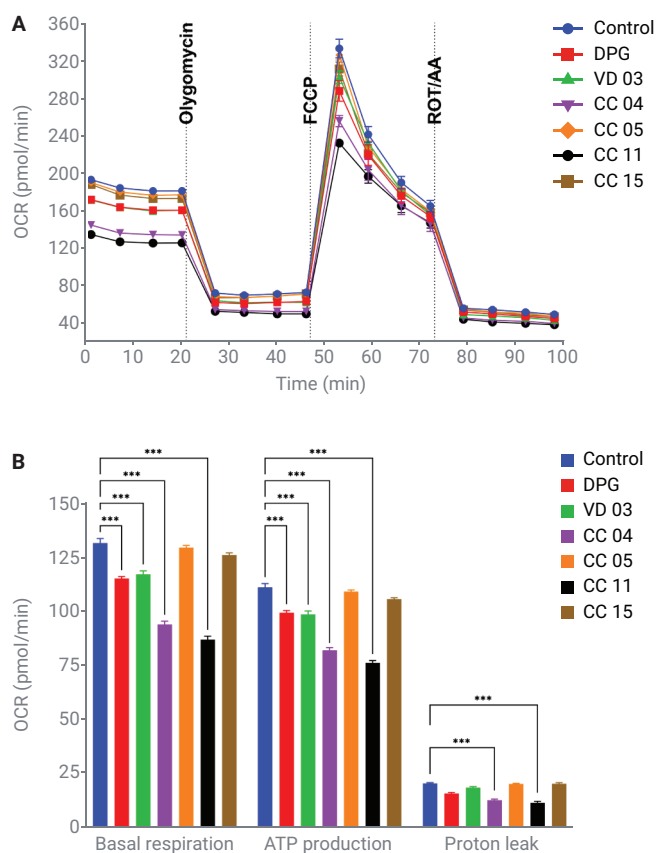
Effects on cell bioenergetics

Though cytotoxicity measurements are essential in evaluating the toxicity of environmental compounds, assessment of subtle effects on cell bioenergetics can provide information about the causes of the toxicity measured.[18] Mitochondrial energy production disruption mechanisms due to chemical exposure include interference with adenosine triphosphate (ATP) synthase, uncoupling of the inner and outer mitochondrial membranes (which interferes with the proton gradient required for ATP production), and inhibition of the protein complexes that create the electron transport chain's (ETC) electrochemical gradient. The Seahorse XF96 analyzers provides this metabolic data in live cells in real time. The Seahorse XF96 analyzer results shown in Figure 4 demonstrate that, except for CC05, all of the DPG compounds tested had an adverse effect on cell bioenergetics. While the compounds' more limited effects on mitochondrial basal respiration and ATP production could have been short lived, the relatively significant effect on proton leakage suggested irreversible mitochondrial damage.
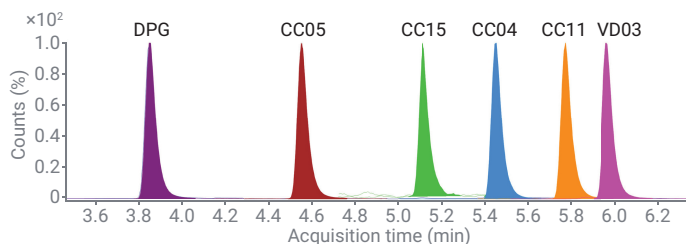
DPG chlorination and chloramination product formation in water

The 6495C Triple Quadrupole LC/MS MRM method provided good separation and detection of DPG and its target chlorination products (Figure 5). The calibration curves were linear with R^2 values of 0.99 and above over the concentration range of 0.0025 to 10 ng/mL.

As shown in Figure 6, when DPG was reacted with free chlorine at concentrations ranging from 7 µM to 80 µM for 10 minutes, the lower doses up to 40 µM favored the formation of the di-halogenated products in the order CC15 > CC05 > CC04. CC11 and VD03, the tri-halogenated and tetra-halogenated products, respectively, were not detected. At lower molar ratios of free-chlorine, DPG, CC15, CC05, and CC04 accounted for up to 42% of the products formed (Cl_2:DPG = 2.8). At higher chlorine doses, the target products accounted for about 10% of the initial DPG concentration, indicating the occurrence of additional transformation products. The target chlorinated products (CC04, CC05, CC11, CC15, and VD03) were not observed in samples taken during the chloramination experiments.

Figure 4. Results from cell bioenergetics and mitochondrial stress tests. (A) mitochondrial stress test profile of A549 cells treated with 5 µM test samples and controls for 24 hours. The OCR was measured following addition of oligomycin (an ATP synthase inhibitor), FCCP (a protein gradient uncoupler, FCCP stimulates the respiratory chain to operate at maximum capacity, increasing substrate oxidation), and rotenone/antimycin A (an electron transport chain disruptor) at the time points shown. (B) Basal respiration, a measurement of the cells relative mitochondrial respiration and glycolysis under resting conditions per compound tested. ATP production rates are reflected by the difference in OCR levels before and after oligomycin addition per compound tested. Proton leak reflects the remaining basal respiration not coupled to ATP production. Data expressed as mean ±SD (n = 6). *** denotes p <0.001; from DMSO control using one-way ANOVA analysis.

Figure 5. MRM chromatogram of a 100 ppt mix of DPG and the chlorination products CC04, CC05, CC11, CC15, and VD03.
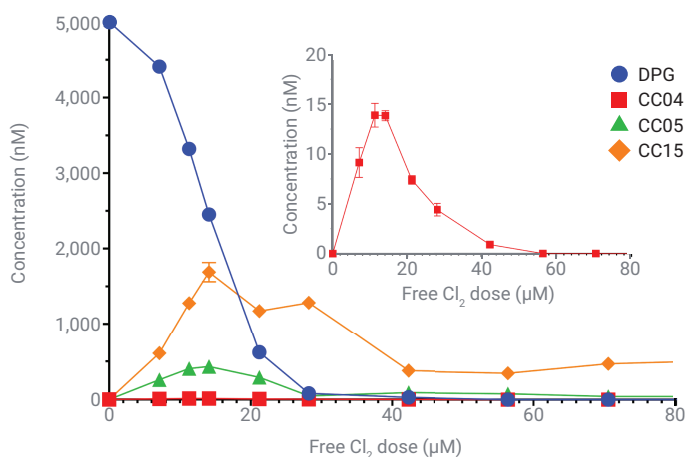
Figure 6. Concentration of DPG and selected products during chlorination experiments measured by triple quadrupole LC/MS. Data points are for three replicate samples.

Even though CC05 and CC15 resulted in lower cytotoxicity compared to the other products tested in the RTCA and mitochondrial stress tests, the higher concentrations produced during the simulated chlorination experiments (400 to 1,500 nM) are a concern, particularly for chlorinated waters in that could come in direct contact with pipes or other components capable of leaching DPG. Although CC04 was only formed at low concentrations (up to 13.8 nM), its toxic potential is also of concern. The concentration of DPG used in the simulated chlorination experiments (5 µM / ~1 mg/L) is in the range described in previous studies that reported DPG leaching from HDPE water pipes (up to 0.74 mg/L).[9] Reported environmental concentrations in surface waters range from 5 ng/L to more than 500 ng/L.[5]

Conclusion

DPG is a major component released from tires during wear and aging and from HDPE pipes used in municipal water systems. Though DPG is found in waters around the world at low ng/L to mg/L concentrations, its effects on environmental health are not fully understood. This application note evaluated DPG and five of its chlorinated products' effects on cell heath and bioenergetics using xCELLigence RTCA eSight and Seahorse XF96 extracellular flux analyzer *in vitro* assays. RTCA analyses were compared to traditional alamarBlue assays.

The alamarBlue assay indicated that C11 is the most and CC15 is the least cytotoxic DPG product. Compared to the cytotoxicity of DPG, only CC11 produced lower EC_{50} values in the A549 cell line, suggesting it is more toxic than DPG itself. Results of the real-time cellular impedance from the xCELLigence RTCA eSight were similar in the initial 24 hours

of exposure to DPG and its chlorination products at 5 µM. In this case, CC11, CC04, and DPG had the most impact on cell viability. However, after 168 hours of exposure, all of the chlorinated products tested were more toxic than DPG. The order of toxicity from lowest to highest was CC11 > CC04 > VD03 > CC05 > CC15 > DPG. The automated, label-free, real-time nature of xCELLigence RTCA eSight assays simplified the examination of toxicity effects over various time windows. Assessment of the compounds' effects on cell bioenergetics using Seahorse XF96 extracellular flux analyzer demonstrated that, except for CC05, all of the compounds tested had an adverse effect, affecting cellular basal respiration rates and ATP production. In addition, DPG and its two chlorination products, CC04 and CC11 had an impact on mitochondrial proton leak, which is an indicator of mitochondrial damage.

After evaluating the compounds' real-time effects on cell health and bioenergetics, triple quadrupole LC/MS MRM experiments were used to characterize the formation of compounds produced by reaction of DPG with chlorine and monochloramine under conditions mimicking those found in water treatment and distribution networks. Lower chlorination doses up to 40 µM favored the formation of the di-halogenated products in the order CC15 > CC05 > CC04. CC11 and VD03, the tri-halogenated and tetra-halogenated products, respectively, were not found. At lower molar ratios of free-chlorine, DPG, CC15, CC05, and CC04 accounted for up to 42% of the products formed (Cl_2:DPG = 2.8). At higher chlorine doses, the target products accounted for about 10% of the initial DPG concentration, indicating the occurrence of additional transformation products. The target chlorinated products were not observed in samples taken during the chloramination experiments.

When examining the simulation results together with cell toxicity and cell bioenergetics measurements, the high concentrations of CC05 and CC15 produced during the simulated chlorination experiments (400 to 1,500 nM) are a concern, particularly for chlorinated waters that come in direct contact with pipes or other components capable of leaching DPG. Though CC04 was only formed at low concentrations (up to 13.8 nM), its potential for toxicity is of significant concern due to its effects on cell viability and cell bioenergetics.

The combination of *in vitro* cytotoxicity and cell bioenergetics assays evaluated with 6495C Triple Quadrupole LC/MS characterization of simulated by-product formation offers a workflow applicable to environmental toxicology screening early in product development with the goal of reducing hazardous chemical release into the environment.

Acknowledgments

References

1. Jin, J. *et al.* On the Various Roles of 1,3-DIPHENYL Guanidine in Silica/Silane Reinforced sbr/br Blends. *Polym. Test.* **2021**, *93*, 106858.

2. Wik, A.; Dave, G. Occurrence and Effects of Tire Wear Particles in the Environment – a Critical Review and an Initial Risk Assessment. *Environ. Pollut.* **2009**, *157(1)*, 1–11.

3. Scheurer, M. *et al.* Persistent and Mobile Organic Chemicals in Water Resources: Occurrence and Removal Options for Water Utilities. *Water Supply* **2021**, *22(2)*, 1575–1592.

4. Schulze, S. *et al.* Occurrence of Emerging Persistent and Mobile Organic Contaminants in European Water Samples. *Water Res.* **2019**, *153*, 80–90.

5. Zahn, D. *et al.* Identification of Potentially Mobile and Persistent Transformation Products of REACH-Registered Chemicals and Their Occurrence in Surface Waters. *Water Res.* **2019**, *150*, 86–96.

6. Johannessen, C. *et al.* The Tire Wear Compounds 6PPD-quinone and 1,3-diphenylguanidine in an Urban Watershed. *Arch. Environ. Contam. Toxicol.* **2022**, *82(2)*, 171–179.

7. Hou, F. *et al.* Quantification of Organic Contaminants in Urban Stormwater by Isotope Dilution and Liquid Chromatography-Tandem Mass Spectrometry. *Anal. Bioanal. Chem.* **2019**, *411(29)*, 7791–7806.

8. Xie, L. *et al.* Simultaneous Screening for Chemically Diverse Micropollutants in Public Water Bodies in Japan by High-Performance Liquid Chromatography–Orbitrap Mass Spectrometry. *Chemosphere*. **2021**, *273*, 128524.

9. Tang, J. *et al.* Different Senescent HDPE Pipe-Risk: Brief Field Investigation from Source Water to Tap Water in China (Changsha City). *Environ. Sci. Pollut. Res.* **2015**, *22(20)*, 16210–16214.

10. Dejonckheere, G. *et al.* Allergic Contact Dermatitis Caused by Synthetic Rubber Gloves in Healthcare Workers: Sensitization to 1,3-diphenylguanidine is Common. *Contact Dermat.* **2019**, *81(3)*, 167–173.

11. Stalter, D. *et al.* Mixture Effects of Drinking Water Disinfection By-Products: Implications for Risk Assessment. *Environ. Sci.: Water Res. Technol.* **2020**, *6(9)*, 2341–2351.

12. Xie, S. H. *et al.* DNA Damage and Oxidative Stress in Human Liver Cell L-02 Caused by Surface Water Extracts During Drinking Water Treatment in a Waterworks in China. *Environ. Mol. Mutagen.* **2010**, *51(3)*, 229–235.

13. Yin, J. *et al.* Comparative Toxicity of Chloro- and Bromo-Nitromethanes in Mice Based on a Metabolomic Method. *Chemosphere*. **2017**, *185*, 20–28.

14. Hrudey, S. E. *et al.* Evaluating Evidence for Association of Human Bladder Cancer with Drinking-Water Chlorination Disinfection By-Products. *J. Toxicol. Environ. Health, Part B.* **2015**, *18(5)*, 213–241.

15. Sieira, B. J. *et al.* Chlorination and Bromination of 1,3-diphenylguanidine and 1,3-di-o-tolylguanidine: Kinetics, Transformation Products and Toxicity Assessment. *J. Hazard. Mater.* **2020**, *385*, 121590.

16. Mauricius Marques dos Santos *et al.* Genotoxic Effects of Chlorinated Disinfection By-Products of 1,3-diphenylguanidine (DPG): Cell-based *In Vitro* Testing and Formation Potential During Water Disinfection. *J. Hazard. Mater.* **2022**, Volume 436, 2022, 129114, ISSN 0304-3894, https://doi.org/10.1016/j.jhazmat.2022.129114.

17. Ramis, G. *et al.* Optimization of Cytotoxicity Assay by Real-Time, Impedance-Based Cell Analysis. *Biomed. Micro.* **2013**, *15(6)*, 985–95.

18. Zuo, Y. T. *et al.* Toxicity of 2,6-dichloro-1,4-benzoquinone and Five Regulated Drinking Water Disinfection By-Products for the Caenorhabditis Elegans Nematode. *J. Hazard. Mater.* **2017**, *321*, 456–463.

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