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WATER PRODUCTION FOR AQUAPONICS V.1

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We use this protocol and it's working

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Abstract

The protocol summarises the procedures used for analytical control – detailed protocols are annexed to this protocol.

Guidelines

RECOMMENDED/ACCEPTED VALUE

#Step2.1: Mesophilic Bacteria in PCA (Plate Count Agar):
According to drinking water EU legislation

#Step2.2: Total Coliforms, Fecal Enterocci and Escherichia Coli:
Total coliforms: 0 MPN/ mL
E.coli: 0 MPN/ mL
Fecal enterococci: 0 MPN mL

#Step2.3 Total Bacteria and Antibiotic Resistance Genes:
No legal recommendation available.
16S rRNA gene <3 log units/ mL ; other faecal and antibiotic resistance genes < limit of detection (LOD) in 2000 mL

#Step2.4: Virus:
Viruses not included in any normative.
Contemplated in the EU proposal of a new directive on the Treatment of urban wastewater

#Step2.5: Parasites:
Regulation 2020/741 Minimum requirements for water reuse.
Guidelines to support the application of Regulation 2020/741 on minimum requirements for water reuse
Council Directive 93/88/EEC of 12 October 1993 on the protection of workers from risks related to exposure to biological agents at work.
Relevant food-borne parasites are not included in any normative.

#Step2.6: Metals:
Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy.

#Step2.7: Organic Contaminants:
Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality standards in the field of water policy.

Materials

	A	B	C	D	E	F	G	H
	Parameter	V (mL) x R	S	Processing	Analytical method	Result	LOD / LOQ	Goal value
	Mesophilic Bacteria in PCA (Plate Count Agar)	0.1 x 3	No	Spread method	Spread plate; 48 h incubation at 37 °C	Number of Colony Forming Units (CFU/mL)	10	0 CFU/mL
	Total coliforms, Fecal enterococci and Escherichia coli	100 x 3	No	None	Colilet 18 system (IDEEX) method – Total coliforms and E.coli Enterolert E system (IDEEX) method – Fecal enterococci	Most Probable Number (MPN/mL)	1	0 MPN /100 mL
	Total bacteria and antibiotic resistance genes	2000 x 3	On ice	Membrane filtration 0.22 µM polycarbonate membranes	DNA-based analysis quantitative PCR (e.g. 16S rRNA;intI1, uidA, sul1, qacE1, tetX, ermB, crassphage, mefC, ermF, aph(3´´)-ib	Gene abundance per volume of water (Log-unit / mL) Removal values (if adequate) (Log-unit)	Total bacteria: 0.7 Other genes: intI1 – 0.01 uidA – 0.01 sul1 – 1.22 qacEΔ1 – 0.2 tetX – 0.2 ermB – 0.2 crassphage – 1.0 mefC – 0.1 ermF – 0.01 aph(3´´)-ib – 1.20	Total bacteria (3 log-units / mL) Other genes (
	Viral concentration	5000 x 1	On ice	Filtration with Rexeed dialysis units, concentration with aluminium chloride	Reverse transcription quantitative PCR	Viral abundance (GC, Gene-copy / L) Removal values (GC, Gene-copy /L)	SaV: LOD 7.7x 10(3) / LOQ 1.2x10(4) NoV GI: LOD 1.3x103/ LOQ 1.7 x103() NoV GII: LOD 3.7 x10(3)/ LOQ 8.5x10(3) HEV: LOD 2.1x104 / LOQ 6.010(4) HAV: LOD 2.6 x10(2) / LOQ 3.2 x10(2)	Viruses < LOD
	Parasite	250 x 12	On ice	Membrane Filtration Nitrocellulose/polycarbonate membranes of 0.45 µM	High throughput sequencing	Presence/absence (Amplicon Sequence Variants, ASVs) of hazardous species (potential human	Acremonium sclerotigenum - 341 Aspergillus versicolor - 20 Cladosporidium sp. - 1101 Penicillium sp. -	ASV < LOD

	A	B	C	D	E	F	G	H
						pathogens: hypersensitive pneumonitis, asthma, allergy, immunosuppressive clinical symptoms and rhinitis, Legionella spp. and its host; fish pathogen)	27 Naegleria sp. - 240 Alternaria sp.- 122 Cryptocarion sp. - 56 Legionella sp. - 10 Rhogostoma sp.- 130	
	Metals	50 x 2	On ice	Acidification (0.15M HNO3)	ICPM-MS	Metals quantity (parts per billion (ppb) / parts per trillion (ppt)	Limit of Detection Al – 0.1 ppb V – 0.01 ppb Cr – 0.001 ppb Mn – 0.01 ppb Fe – 0.05 ppb Co – 0.001 ppb Ni – 0.01 ppb Cu – 0.01 ppb Zn – 0.05 ppb Th – 0.01 ppt Cd – 0.0005 ppb Csi – 0.005 ppb Ba – 0.01 ppb Pb – 0.005 ppb As – 0.01 ppb Sn – 1.0 ppt Sb – 1.0 ppt Pt – 0.1 ppt Tl – 0.01 ppt Bi – 0.01 ppt U – 0.01 ppt Ag – 0.01 ppt Hg – 0.01 ppt La – 0.01 ppt Ce – 0.01 ppt Pr – 0.001 ppt Nd – 0.01 ppt Sm – 0.005 ppt Eu – 0.005 ppt Gd – 0.01 ppt Tb – 0.005 ppt Dy – 0.005 ppt Ho – 0.005 ppt Er – 0.005 ppt Tm – 0.005 ppt Yb – 0.005 ppt Lu – 0.005 ppt	
	Organic contaminants	500 x 2	On ice	Solid-phase extraction	HPLC/MS-TOF	Presence/absence	-	Not present

Table 1: Samples, Processing and Analysis of the different parameters analysed.
V, volume; R, Replicates; S, Shipment conditions; LOD / LOQ, Limit of Detection / Quantification

#Step2.1 Materials for Mesophilic Bacteria in PCA (Plate Count Agar): Culture medium Plate Count Agar (PCA); Microbiological incubator; other microbiology consumables.

#Step2.2 Materials for Total coliforms, Fecal enterococci and Escherichia coli:

#Step2.2 Sterile bottles, IDEEX kit, Colilert-18 reagent, Microbiological incubator.

#Step2.3 Materials for Total Bacteria and Antibiotic Resistance Genes: Membrane filtration (nitrocellulose 0.22 µm pore); DNA extraction (QIAGEN, Power Water); Quantitative PCR of genes such as 16S rRNA gene (total bacteria), *uidA*, *marA* (bacterial contaminant indicators), *crassphage* (human fecal contamination), *intI1* / *incF* / *sul1* / *qacEΔ1* / *tetX* / *ermB* / *mefC* / *ermF* / *aph(3´´)-ib* (antibiotic resistance indicators); metagenomics analysis of selected samples.

#Step2.4 Materials for Virus Rexeed 25A filtration units (Asahi Kasei Medical); other molecular biology consumables.

#Step2.5 Materials for Parasites Filtration ramp; DNA extraction kit, PCR equipment; fluorimeter for DNA quantification; outsourcing of sequencing service; molecular biology consumables.

#Step2.6 Materials for Metals Metal-free centrifuge tubes

#Step2.7 Materials for Organic Contaminants

	A	B	C	D
		Becker		cartridges (OASIS HLB 200 mg)
	Pre-filteration	Buchner flask	**SPE**	vacuum pump
		PVDF 0.45 µm filters		graduated cylinder
		trap flask		becker
		vacuum pump		trap flask
				SPE vacuum manifold system

Safety warnings

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1 The water production for AWARE main activities includes three stages – disinfection by ultraviolet C radiation (UVC), storage for 12:00:00 - 24:00:00 (according to water load and season) and ozonation. The water quality is monitored at these three stages, for the parameters indicated in Figure 1 below. 1d 12h

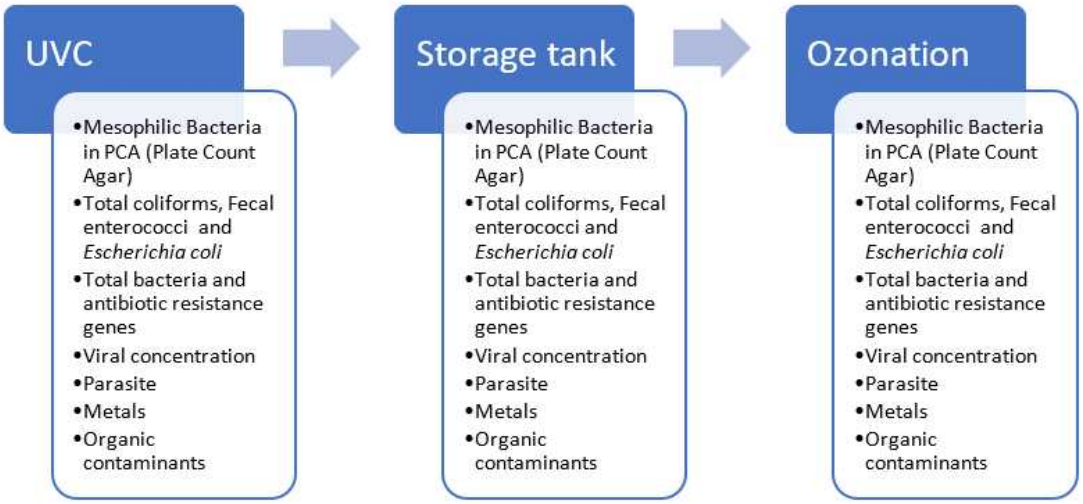

Figure 1. Treatment and storage of municipal treated wastewater used for integrated aquaponics and an indication of the comprehensive quality and risk assessment.

1.1 Sampling, Processing, and Analyses

9h

Water samples are collected (see Figure 2) and processed within a 06:00:00 interval, before being shipped for the partner responsible for the analyses (Table 1). In case no processing is needed, samples are frozen and stored at -80 °C within 03:00:00 .
For each sampling event, the date, day of the week and hour; the temperature and rain.
Sampling points, indicated in Figure 2 were designated from A to I:
- Influent of primary treatment (A)
- Influent of biological treatment (activated sludge) (B)
- Treated secondary effluent (C)
- Sand filter effluent (D)
- UVC effluent (E)
- Storage for reuse tank effluent (F)
- Ozonation effluent (1 dose, e.g., 5 mg O_3) - MITO3X technology - (G)
- Effluent of the vacuum UV oxidation (VUV) (H)
- Effluent of reactive storage / Influent of the recirculation aquaculture system (RAS) (I)

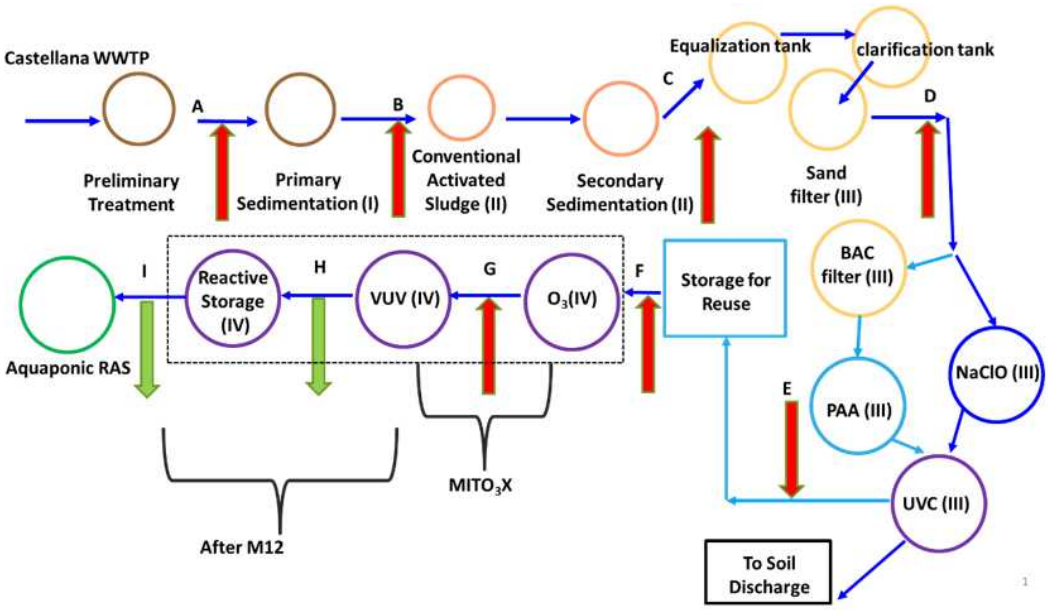

Figure 2. Diagram representing the wastewater treatment plant (WWTP), advanced treatment and sampling points.

Methods: The section below summarises the procedures used for analytical control – detailed protocols are annexed to this protocol. 5d

2 **Mesophilic Bacteria in PCA (Plate Count Agar):**

2.1 **Analysis:** Enumeration of culturable mesophilic bacteria at 37 °C 2d 12h

Materials: Culture medium Plate Count Agar (PCA); Microbiological incubator; other microbiology consumables.

Method:

Spread plate; units 48:00:00 a incubation at 37 °C ; emuneration of colony forming (CFU/ mL):

Observations: Samples processed and analysed within 12:00:00 after collection.

2.2 **Total Coliforms, Fecal Enterococci and *Escherichia coli:*** 12h

Analysis: Enumeration of fecal contamination indicators.

Material: Sterile bottles, IDEEX kit, Colilert-18 reagent, Microbiological incubator.

Method:

	A	B
	Total coliforms E.coli	**Colilet 18 (IDEEX) method**
	1	100 mL of each water sample poured in sterile bottles from IDEEX kit
	2	Adding of Colilert-18 reagent
	3	Mix sample + reagent poured into the QuantiTray/2000 System
	4	Incubation 18 hours, 35 °C ± 0.5 °C
	5	Results analysis

Reference: go to step #2.3

− UNI EN ISO 9308-2:2014 Water quality - Count of *Escherichia coli* and coliform bacteria - Part 2: Most probable number method.
− EN ISO 11133:2014 - Microbiology of food, animal feed and water. Preparation, production, storage and performance testing of culture media.

	A	B
	Fecal enterococci	Enterolert E system (IDEEX)
	1	100 mL of each water sample poured in sterile bottles from IDEEX kit
	2	Adding of reagent
	3	Incubation 24 hours, 41 °C ± 0.5 °C
	4	Results analysis

Reference: go to step #2.3

− UNI EN ISO 7899-1:2001 - Water quality - Research and enumeration of intestinal enterococci - Miniaturized method (Most Probable Number) for surface and wastewater.
− EN ISO 11133:2014 - Microbiology of food, animal feed and water. Preparation, production, storage and performance testing of culture media.

Observations: Samples were processed and analysed within 12:00:00 after collection.

2.3 Total Bacteria and Antibiotic Resistance Genes:

6h

Analysis: Culture-independent detection and/or quantification of bacteria and bacterial contaminants.

Material: Membrane filtration (nitrocellulose 0.22 µm pore); DNA extraction (QIAGEN, Power Water); Quantitative PCR of genes such as 16S rRNA gene (total bacteria), *uidA*, *marA* (bacterial contaminant indicators), *crassphage* (human fecal contamination), *intI1* / *incF* / *sul1* / *qacEΔ1* / *tetX* / *ermB* / *mefC* / *ermF* / *aph(3´´)-ib* (antibiotic resistance indicators); metagenomics analysis of selected samples.

Observations: Samples were filtered within 06:00:00 after collection the filtering membranes were immediately frozen and stored at -80 °C till shipping in dry ice to the respective partner who proceeded for DNA extraction.

2.4 Virus:

6h

Analysis: Detection and enumeration of virus.

Material: Rexeed 25A filtration units (Asahi Kasei Medical); other molecular biology consumables.

Method: Virus capture by filtration with Rexeed dialysis units, concentration with aluminium chloride, nucleic acid extraction and detection by RT-qPCR.

Observations: Samples were filtered within 06:00:00 after collection the filtering membranes were immediately frozen and stored at -80 °C till shipping in dry ice to the respective partner who proceeded for DNA extraction.

2.5 Parasites:

1d 12h 15m

Analysis: Detection of parasites.

Material: Filtration ramp; DNA extraction kit, PCR equipment; fluorimeter for DNA quantification; outsourcing of sequencing service; molecular biology consumables.

Method:
Processing of Water samples

1. Filtration.

3 L of water were filtered through 0.45 µm pore size diameter Nitrocellulose / polycarbonate sterile membranes, using a membrane filter holder (47 mm diameter) apparatus. For every approx. 250 mL of water, a new sterile membrane was used to avoid clogging of membranes. Filters corresponding to each litre of water were separated, inserted into well-sealed tubes (50 mL) stored a -80 °C and then transported in dry ice.

2. Centrifugation.(1 L of water was concentrated by centrifugation at 2.500 g for 00:15:00 using sterilized tubes. Pellets obtained were resuspended using of 1 mL of 10 % formaldehyde solution and maintained for 24:00:00 . Pellets resuspended in formaldehyde solution were centrifugated at 2.500 g for , the supernatant was discarded and all pellets were pooled in 1 mL of 70 % ethanol.

3. DNA Isolation, Amplification and sequencing.
DNA extraction was carried out using a commercially available kit (Powerwater kit, Qiagen) in accordance with the manufacturer's guidelines. The V4 and V9 of the 18S rRNA gene hypervariable regions of the eukaryote 18S rRNA gene were amplified using universal primers. Subsequently, PCR products were purified and subjected to external sequencing using Illumina MiSeq platform 300bp paired-end sequencing.

4. Bioinformatic analysis.
Raw sequence data were processed using QIIME2 software 2023.9. Pairedend reads were subjected to quality filtering (FastQC) and denoising (DADA2 plugin). Subsequently, the taxonomic assignment was conducted using the reference database SILVA 18S release 138 with a clustering threshold of 99 % similarity. Alpha rarefaction analysis, Shannon diversity index, richness estimation, and relative abundance calculations were performed using the Vegan R package to assess microbial community diversity and composition particularly focusing on the detection of genetic material from potential pathogens.

Observations: Samples were filtered within 12:00:00 after collection the filtering membranes were immediately frozen and stored a -80 °C till shipping in dry ice to the respective partner for further analysis.

2.6 **Metals:** 12h

Analysis: Detection and quantification of metals

Material: Metal-free centrifuge tubes

Method: Acidified water samples were analyzed for several metals (e.g. Al, Cu, Ni, Co, Pb, Zn, Cd, Pt, Sb, Sn, U, As, Ag, Hg, REEs) by means of ICP-MS (7900 Agilent) using matrix-matched external calibration.

Observations: Samples were filtered/centrifuged within 12:00:00 after collection, and then acidified to 0.15M HNO3.

2.7 **Organic Contaminants:** 1d 0h 30m

Analysis: Screening of Organic Contaminants in Water.

Material: go to step #2.7 Materials for Organic Contaminants

Methods: Solid-phase extraction

1. Sample filtration (0.45um PVDF).

2. Collect 200 mL of the filtered water sample (e.g. volumetric flask or beaker) and spike with 50 µL of an internal standard solution. Produce two 200 mL replicates per sample. Mix well after the internal standard is added.

3. Cleaning/Conditioning of the cartridges (OASIS HLB 200 mg)

3.1. Pass 5 mL of MeOH

3.2. Pass 5 mL of ultrapure water

4. Pass the 200 mL of sample

5. Rinse the volumetric flask or beaker that contained the sample with 2x 10 mL of ultrapure water, which are then passed through the cartridege

6. Drying the cartridge resin (e.g. N2 flow for 00:30:00)

7. Store the cartridges in a freezer (-20 °C)

All samples are to be processed in duplicate.

For the **cartridge blanks** (n=2): start with step 3, and then spike directly on the cartridge the same amount of internal standard as for samples (50 µL mix de 1 ug mL). Then go directly to step 6.
For the **ultrapure water blanks** (n=2), the same type of sampling flasks are filled with ultrapure water 24:00:00 before sampling. Then they are treated using the same protocols as for samples.

Elution of the loaded cartridges was carried out by gravity using 10 mL of methanol followed by 10 mL of dichloromethane. The eluates were concentrated approximately to ca. 0.5 mL using a Turbovap II concentrator (Zymark, Hopkinton, MA, USA), then to dryness under a gentle nitrogen stream and finally reconstituted in 500 µL of methanol and filtered with a 0.2 µm 0 mg GHP® 13 mm syringe filter (Pall Corporation). The extracts were injected in an Agilent 1290 LC coupled with and Agilent 550 QTOF system.

Parameters framed by Legal and Regulatory Requirements: 9h

3 Using the EU Drinking Water Directive:

Mesophilic Bacteria in PCA (Plate Count Agar) – 0 CFU/ 100 mL

Total coliforms and *Escherichia coli* –Number / 100 mL (0 MPN/ 100 mL)

Fecal *enterococci* –Number/100 mL (0 MPN/ 100 mL)

Viral concentration - There are no legal requirements for viruses. They are not included in any regulation now.

Parasite - EU legislation (2020/741)

Metals - DIRECTIVE 2008/105/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on environmental quality standards in the field of water policy

Organic contaminants - DIRECTIVE 2008/105/EC OF THE EUROPEAN PARLIAMENT AND THE COUNCIL of 16 December 2008 on environmental quality standards in the field of water policy.

Protocol references

Mesophilic Bacteria in PCA (Plate Count Agar):

- American Public Health Association (APHA). (2009). Standard Methods for the Examination of Water and Wastewaters. APHA – AWWA - WPCF (Eds.), Pennsylvania, Washington.
- ISO 4833:2003 - Microbiology of food and animal feeding stuff – Horizontal method for enumeration microorganisms – Colony count technique at 30 °C.
- EN ISO 11133:2014 - Microbiology of food, animal feed and water. Preparation, production, storage and performance testing of culture media.

Total Coliforms, Fecal Enterococci and *Escherichia Coli:*

– UNI EN ISO 9308-2:2014 Water quality - Count of *Escherichia coli* and coliform bacteria - Part 2: Most probable number method.
– EN ISO 11133:2014 - Microbiology of food, animal feed and water. Preparation, production, storage and performance testing of culture media.

Total Coliforms, Fecal Enterococci and *Escherichia Coli:*

– UNI EN ISO 7899-1:2001 - Water quality - Research and enumeration of intestinal enterococci - Miniaturized method (Most Probable Number) for surface and wastewater.
– EN ISO 11133:2014 - Microbiology of food, animal feed and water. Preparation, production, storage and performance testing of culture media.

Total Bacteria and Antibiotic Resistance Genes:

– Teixeira *et al.,* 2023 (https://doi.org/10.1016/j.watres.2023.120761)
– Rocha *et al*., 2020 (https://doi.org/10.1016/j.jece.2018.02.022)

Virus:

– **http://aem.asm.org/content/75/16/5284.long;**
– **https://www.miteco.gob.es/es/agua/temas/concesiones-yautorizaciones/vertidos-de-aguas-residuales/alerta-tempranacovid19/vatar-covid19-protocolo-deteccion-sars-cov-2aguas.html**

Parasites:

- Mthethwa N.P., Amoah I.D.,
Reddy P., Bux, F., Kumari S. 2021. A review of the application of next-generation sequencing methods for profiling of protozoan parasites in water: Current methodologies, challenges, and perspectives. Journal of Microbiological Methods, 187:106269
- Ríos-Castro R., Romero A., Aranguren R., Pallavicini A., Banchi E., Novoa B., Figueras A. 2021. High Throughput Sequencing of Environmental DNA as a Tool for Monitoring Eukaryotic Communities and Potential Pathogens in a Coastal Upwelling Ecosystem. Frontiers in veterinary science, 8, 765606.
EFSA (2018). Public health risks associated with food-borne parasites. EFSA Journal 16 (12):5495.

Metals:

U.S. EPA. 1994. “Method 200.8: Determination of Trace Elements in Waters and Wastes by Inductively Coupled Plasma-Mass Spectrometry,” Revision 5.4. Cincinnati, OH.

Organic Contaminants:

Montes, R.; Méndez, S.; Carro, N.; Cobas, J.; Alves, N.; Neuparth, T.; Santos, M.M.; Quintana, J.B.; Rodil, R. Screening of Contaminants of Emerging Concern in SurfaceWater and Wastewater.
Effluents, Assisted by the Persistency-Mobility-Toxicity Criteria.
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