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Genotoxicity and biodegradation of quaternary ammonium salts in aquatic environments

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ABSTRACT

Biodegradation tests were conducted for three groups of quaternary ammonium salts (QAS) that differed in hydrophobic chain length or in hydrophilic properties. The degradation rate was influenced by the hydrocarbon chain length, the presence of aromatic or cyclic rings, and the occurrence of sulphur and oxygen atoms in the alkyl substituent. All tested QAS variants were biodegradable in an aquatic environment. The half life of the different QAS under these conditions ranged from 0.5 to 1.6 days and depended on the properties of the compound. Biodegradation intermediate products were identified by nuclear magnetic resonance spectrometry (^{1}H NMR and ^{13}C NMR). Both the initial preparations and their biodegradation products were not genotoxic.

1. Introduction

Quaternary ammonium salts (QAS) are molecules with at least one long, hydrophobic alkyl chain attached to a positively charged nitrogen atom. QAS are cationic surfactants that are widely used in industry, biotechnology, medicine, pharmacology and in biocide production. They are also used as active ingredient of many cosmetics. They have antimicrobial, fungicidal, algaecidal, antielectrostatic and anticorrosive properties [1–4].

Due to the wide spread use of QAS, leakage into aquatic environments occurs with rain and wastewater from municipal and industrial sources. QAS can harm organisms that live in water and affect both animals and plants. There is also a possibility that the salts can undergo biological and chemical degradation. These substances, considered harmful to human health, have been found in surface water at concentrations up to 3.8 $\mu g\,L^{-1}$ [5]. There is evidence that QAS have genotoxic properties and interact with DNA molecules [6–8]. Consequently, studies on genotoxicity and biodegradation of newly synthesised QAS in aquatic environments are of great interest.

2. Experimental

2.1. Compounds

Three groups of quaternary ammonium salts were investigated. They differed from each other in hydrophobic chain length or hydrophilic properties. Properties of the substances used in the study are given in Table 1.

Three groups of ten compounds that were tested are classified as A, B and I (Table 1):

(A) alkylalkoxymethylammonium chlorides
(B) alkylbenzyldimethylammonium chlorides, and
(C) imidazolium chlorides.

The compounds were prepared by Professor Juliusz Pernak's group (University of Technology, Poznań, Poland).

All compounds were soluble in well water at the investigated concentrations and contained over 95% active molecules. Group A and I compounds act strongly against microorganisms. They possess antiseptic, fungicidal and antielectrostatic properties [4,9,10]. Group B compounds are used as a coating in galvanic baths during nickel plating and zinc plating. Group I chlorides have also recently been used as fungicides for wood protection.

2.2. Biodegradation tests

Laboratory tests on QAS biodegradation were conducted using a modified version of the Simulation Test described in the Orga-

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Table 1
Symbols and molecular weights of tested quaternary ammonium compounds.

Code	Name of compound	Molecular weight ($g\,mol^{-1}$)
A-1	Octyldimethyldecyloxymethylammonium chloride	363.5
A-2	Dodecyldimethyloctyloxymethylammonium chloride	391.5
A-3	Dodecyldimethylnonyloxymethylammonium chloride	405.0
A-4	Dodecyldimethyldecyloxymethylammonium chloride	419.0
B-1	Benzyldimethyloctyloxymethylammonium chloride	313.5
B-2	Benzyldimethyldecyloxymethylammonium chloride	341.5
B-3	Benzyldimethyldodecyloxymethylammonium chloride	369.5
I-1	1–Decyl-3-hexyloxymethylimidazolium chloride	358.5
I-2	1–Decyl-3-cyklohexyloxymethylimidazolium chloride	356.5
I-3	1–Decyl-3-hexylthiomethylimidazolium chloride	374.5

nization for Economic Cooperation and Development (OECD) Guidelines [11].

Assays were conducted under conditions that simulate a natural aquatic environment. However, instead of 1.0 L conical flasks, 40 L glass tanks were used. Such a large quantity of water was required for the analysis of degradation products by (^{1}H and ^{13}C NMR). Tanks were filled with settled tap water and inoculated with activated sludge microorganisms at a concentration of 1 ml L^{-1}. Each QAS variant was then added at a initial concentration of 1 mg L^{-1}. Cultures were aerated because shaking was technically impossible. No foaming was observed during aeration of the water in the glass tanks. The QAS biodegradation test was performed under conditions where the substrates were the only source of carbon and energy for a mixed population of microorganisms.The population of microorganisms used had been previously adapted to degrade the substrates. The cultures were grown at 20 ± 2 °C in the, dark.

The initial concentration was the result of the following issues:

- investigated preparations appeared to be very toxic to aquatic microflora,
- concentrations of QAS appearing in surface waters ranging from 0.1 to 3.8 μg L^{-1} [5].

2.3. Genotoxicological tests

Genotoxicological tests were performed using *Bacillus subtilis* H17 Rec$^+$ and M45 Rec$^-$ strains described by Kada et al. [12]. The strains were cultivated in B-2 bouillon for 24 h and then sieved into a Petri dish containing the same media solidified with agar. A sterile, 1 cm paper disc soaked with each compound was placed at the centre of each dish. *B. subtilis* strains were incubated at 4 °C for 24 h and then at 37 °C for the next 24 h. After 48 h of incubation, the diameter of single colonies for both strains was measured and compared with that of growth controls. Based on the differences in size, each tested compound was assigned to one of three different classifications: not genotoxic (−), with a difference in diameter equal to = 2 mm; potentially genotoxic (±), with a difference in diameter between >2 and 4 mm; and genotoxic (+) with a difference larger than >4 and 6 mm. QAS concentrations varied between 0.1 mg and 10 mg of active ingredient per litre.

B. subtilis strains H17 Rec$^+$ and M45 Rec$^-$ used in the test were obtained from the Department of Microbiology, Faculty of Biology and Environmental Protection at, Silesian University in Katowice, Poland.

2.4. Analytical methods

2.4.1. Disulphine blue analysis

Concentrations of QAS were measured by a conventional disulphine blue active substance test (DBAS) [13]. Briefly, this method is a spectrophotometric test based on the formation of a chloroform soluble blue complex of the cationic surfactant with the anionic dye, disulphine blue. The sensitivity of the DBAS test is 0.01–0.02 mg L^{-1}.

2.4.2. ^{1}H NMR and ^{13}C NMR spectrum analysis

^{1}H and ^{13}C NMR were used for the identification of QAS and their degradation products.

The volume of collected samples was 10.0 L. The samples were extracted with chloroform three times, and the extracted samples were concentrated under vacuum, resulting in a solid residue. The extracts were concentrated in two stages, using 1000 ml and 25 ml flasks in succession. The solid residue obtained was dissolved in 1 ml of deuterated chloroform ($CDCl_3$). ^{1}H and ^{13}C NMR spectra were recorded with a Varian Model XL 300 MHz Spectrometer in DMSO-d_6 or $CDCl_3$, at 20 °C with tetramethylsilane as a standard.

2.4.3. DOC analysis

The concentration of dissolved organic carbon (DOC) in filtered culture medium was determined with a Beckman Industrial Model 915B Tocamaster total organic carbon analyser.

2.5. Interpretation of biodegradation results

The biodegradation of the compounds in "river water" was measured as a function of time and evaluated by statistical methods. For most QAS, the degradation process in water proceeded via first-order kinetic; the concentration decreased exponentially with time:

$$\frac{dC}{dt} = -KC \quad (1)$$

where K is the degradation constant, C is the QAS concentration at time t, and t is the duration of the experiment. The half-life (t_{50}) of the compounds could also be calculated.

$$t_{50} = \frac{\ln 2}{K} \quad (2)$$

3. Results and discussion

The dynamics of QAS degradation differed in individual groups of substrates (Figs. 1–3).

It was found that, among the examined compounds, the most rapid, primary biodegradation occurred for benzyl(alkoxymethyl)dimethylammonium chlorides. After 6 days of incubation, these compounds were no longer present determined by disulfine blue analysis (Fig. 2).

These results confirm previous research that suggests that reports of the negative effects attributed to the biodegradation of QAS compounds containing benzene rings may not be correct [14]. Alkyl(alkoxymethyl)dimethylammonium chlorides were no longer present in water after 7 days (Fig. 1). Cultures containing alkyldimethylimidazolium chlorides had the slowest rate of biodegradation. Trace concentrations of these compounds were

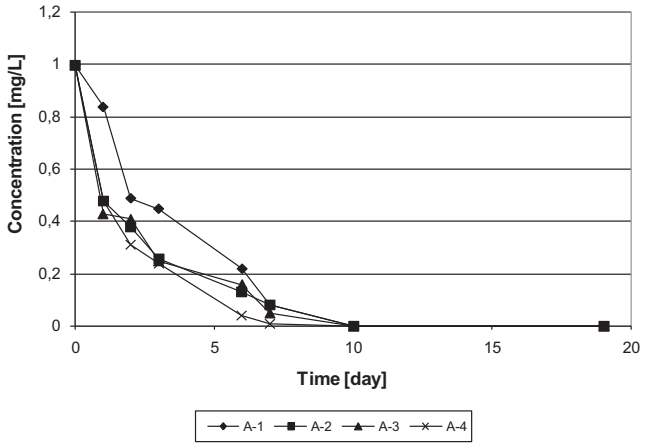

Fig. 1. Changes in concentration of alkyl(alkoxymethyl)dimethylammonium chlorides during biodegradation in the "river-water" test.

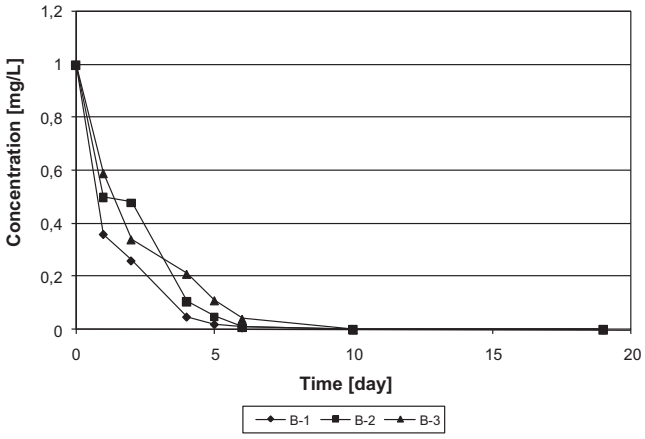

Fig. 2. Changes in concentration of benzyl(alkoxymethyl)dimethylammonium chlorides during biodegradation in the "river-water" test.

found after 10 days of incubation, but a after 19 days, there was no measurable amount based on disulphine blue analysis (Fig. 3).

Analysis of organic compounds by DOC indicated 83.0–93.4% biodegradation depending on the starting QAS (Table 2).

The results presented here are similar to those of French studies that found that alkylimidazolium compounds decay in river water after 7–14 days [15].

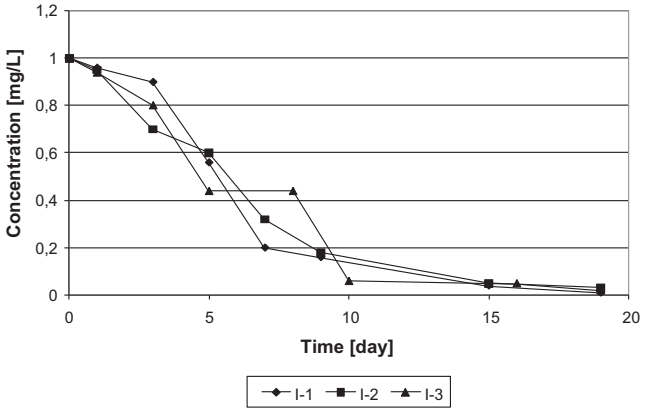

Fig. 3. Changes in concentration of alkyldimethylimidazolium chlorides during biodegradation in the "river-water" test.

Table 2
Biodegradabilities of tested QAS.

Compound	Biodegradability (%) after 19 days	
	DBAS	DOC
A-1	100	87.0
A-2	100	88.0
A-3	100	89.4
A-4	100	90.0
B-1	100	93.4
B-2	100	93.0
B-3	100	89.5
I-1	100	85.8
I-2	100	83.0
I-3	100	84.4

DBAS – disulphine blue active substances, DOC–dissolves organic carbon.

Biodegradation of tested compounds in a model aquatic ecosystem was described by the function $c = f(t)$, where c is the – substance concentration at time t and $f(t) = \ln \frac{C_p}{C_0}$ (Table 3). Correlation coefficients, which ranged from −0.811 to −0.971 and are listed in Table 3, confirm the assumption that degradation of QAS may be estimated based on the first-order equation described above.

Half-life times of eight-carbon chlorides ranged from 0.38 d to 0.52 d (for B-1 and A-4, respectively). Maximum values, up to 0.67 d and 0.81 d, were estimated for compounds that contained twelve carbon atoms (B-3 and A-2) (Table 3). Compound A-1 has the highest antiseptic properties, which probably accounts for the longest measured half-life (0.97 d) in this group of molecules (Table 3) [16]. Values obtained in this study are similar to those presented by Ruiz Cruz and Garcia who have conducted extensive studies about the dependence of the chemical structure of cationic, surface-active agents on biodegradation in river water [17]. They have shown that the half-life of QAS compounds, with 12 carbon atoms in the alkyl chain ranged from less than one day to up to a few days. Nishiyama has suggested that biological oxidation velocity of group A compounds was two, or even three, times slower than for group B and I preparations, which was probably caused by their high antiseptic properties[18]. Pernak et al. have shown that imidazolium salts have a higher antimicrobial activity than alkyldimethylammonium salts [19]. They also stated that the antiseptic effect was increased when a cycloalkoxymethyl substituent was attached to the molecule. These studies indicated that replacement of one alkyl chain (I-1) with cyclohexane caused an increase half-life time from 1.36 d to 1.69 d (Table 3). Replacement of the oxygen atom (I-1) with a sulphur atom (I-3) had less influence on decreasing the reaction velocity than with the increase of the half-life of the compound.

Values obtained for all the groups of compounds tested were lower than the ones reported by Vives-Rego and Woltering, who showed that the half-life for quaternary ammonium salts ranged from 4 to 14 days [20,21]. Previous adaptation of microorganisms for QAS preparations could be a possible explanation for this discrepancy. It is known that previous adaptation of microorganisms can shorten the half-life by a factor of 14–50 [17].

The half-life of the I-2 compound was the longest biodegradation mechanism of the based on ^{1}H NMR (Fig. 4) and ^{13}C NMR (Fig. 5) analysis.

Analysis of the NMR spectra showed, that degradation of 1-decyl-3-cyclohexyloxymethylimidazolium chloride was correlated to the decomposition of the molecule at the position, where the hydrophobic chain was connected to the nitrogen. As a result of microbiological degradation, intermediate products, such as alcohols and dimethyloamines, were formed. Alcohols were not oxidised and were detected after the biodegradation process. It is assumed, that dimethyloamine was degraded to methyloamine, and this intermediate was further degraded to NH_4^+, which can be used as a nutrient for the microorganisms. After the biodegrada-

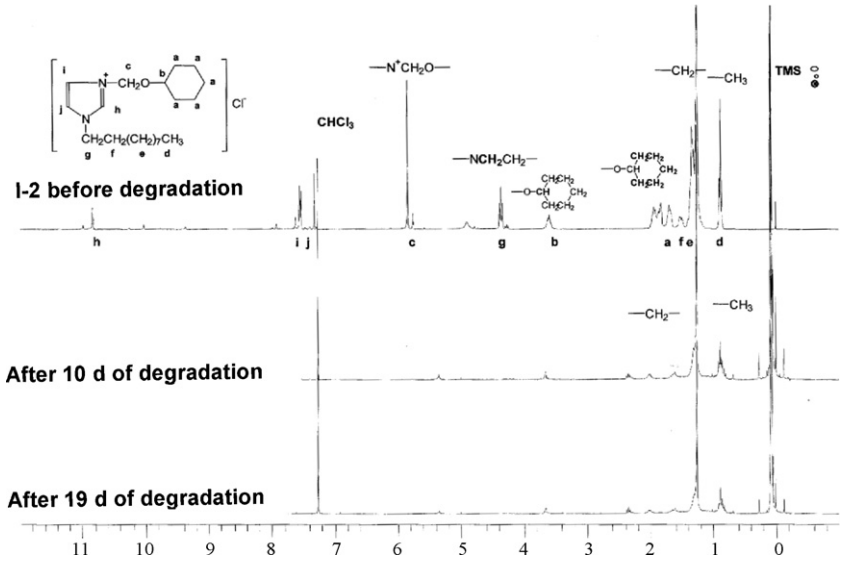

Fig. 4. ^{1}H NMR spectrum for 1-decyl-3-cyclohexyloxymethylimidazolium chloride at different stages of the biodegradation process.

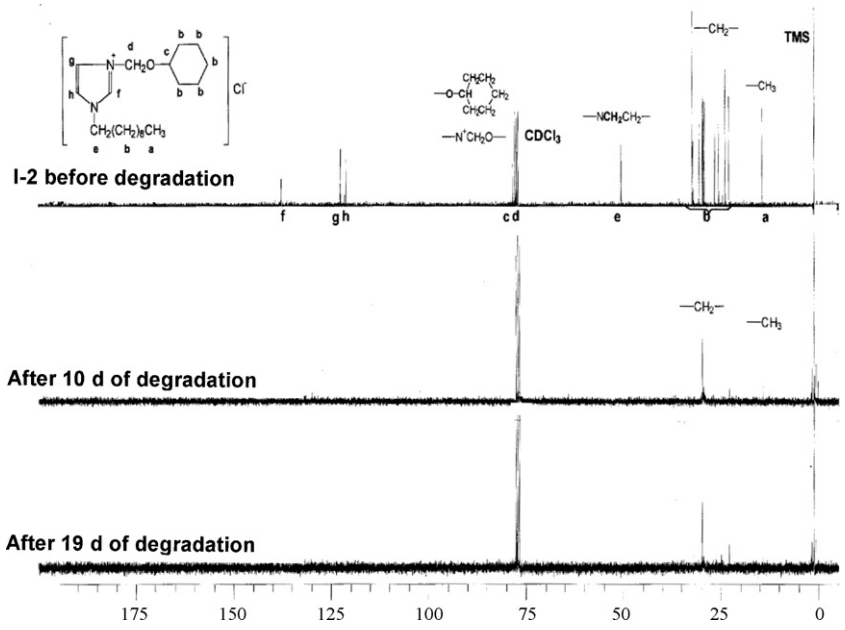

Fig. 5. ^{13}C NMR spectrum for 1-decyl-3-cyclohexyloxymethylimidazolium chloride at different stages of the biodegradation process.

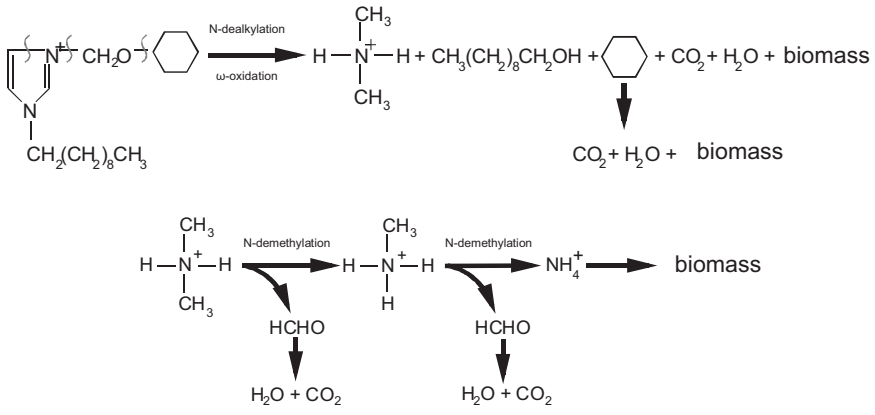

Fig. 6. Hypothetical mechanism of the biological decomposition of 1-decyl-3-cyclohexyloxymethyl imidazolium chloride.

Table 3
Kinetic parameters of reaction and correlation coefficients for tested quaternary ammonium salts.

Quternary ammonium salts group	Compound symbol	Equation $\ln \frac{C_p}{C_0} = f(t)$	Correlation coefficient between C and tR^2	Degradation constant $(1/d)$ $K \pm s$	Half-life time t_{50} (d)
Alkylalkoxymethyldime-thylammonium chlorides	A-1	$-0.31x + 0.093$	−0.912	0.71 ± 0.08	0.97
	A-2	$-0.37x + 0.083$	−0.879	0.85 ± 0.07	0.81
	A-3	$-0.39x + 0.093$	−0.811	0.89 ± 0.07	0.77
	A-4	$-0.59x + 0.063$	−0.956	1.36 ± 0.1	0.50
Benzylalkoxymethyl-dimethylammonium chlorides	B-1	$-0.78x + 0.027$	−0.966	1.8 ± 0.06	0.38
	B-2	$-0.67x + 0.06$	−0.889	1.54 ± 0.14	0.45
	B-3	$-0.45x + 0.10$	−0.830	1.03 ± 0.05	0.67
Alkylmethylimidiazolium chlorides	I-1	$-0.22x + 0.012$	−0.951	0.5 ± 0.04	1.38
	I-2	$-0.18x + 0.023$	−0.971	0.41 ± 0.04	1.69
	I-3	$-0.2x + 0.19$	−0.894	0.46 ± 0.06	1.50

C_p – initial concentration of tested compound, C_0 – concentration of tested compound after time t, t – time, s – standard deviation.

Table 4
Genotoxicological effects of the investigated QAS on *Bacillus subtilis*.

Compound concentration mg L^{-1}	Difference between grow controls [mm]				(−) – non genotoxic properties, (±) – potentially genotoxic properties					
	A-1	A-2	A-3	A-4	B-1	B-2	B-3	I-1	I-2	I-3
0.01	0 (−)	0 (−)	0 (−)	0 (−)	1 (−)	0 (−)	0 (−)	0 (−)	0 (−)	0 (−)
0.1	1 (−)	0 (−)	0 (−)	1 (−)	1 (−)	0 (−)	0 (−)	1 (−)	1 (−)	1 (−)
0.5	1 (−)	0 (−)	0 (−)	0 (−)	1 (−)	0 (−)	0 (−)	1 (−)	1 (−)	1 (−)
1	1 (−)	0 (−)	1 (−)	2 (−)	1 (−)	0 (−)	0 (−)	1 (−)	2 (−)	1 (−)
3	1 (−)	1 (−)	2 (−)	1 (−)	2 (−)	2 (−)	1 (−)	1 (−)	2 (−)	2 (−)
10	2 (−)	2 (−)	2 (−)	2 (−)	2 (−)	3 (±)	1 (−)	1 (−)	2 (−)	2 (−)

tion process, neither the ^1H spectrum or ^{13}C spectrum showed the presence of any protons connected to nitrogen. The cyclohexane ring was presumed to be oxidised to CO_2 and H_2O because, at the end of the process, the aromatic compounds were not present in the solution (Fig. 6). Intermediates were not genotoxic in any of the tests.

Data relating to mutagenic properties are given in Table 4.

The results indicated that tested compounds did not have mutagenic properties. Only the B-2 compound seems to be potentially mutagenic and, even then,only at concentrations 10 mg L^{-1}. Nevertheless, this concentration is much higher than normally occurs in drinking water or natural bodies of water. Cationic surfactant concentrations have been measured between 0.1 and 3.8 μg L^{-1}, in the Tamagawa, Arkawa, Edogawa and Yodogawa Rivers in Japan [5]. There is no monitoring system of QAS concentration in the surface water in Poland. Genotoxicity of the products formed from QAS biodegradation was also investigated in this study, and no toxic effect was found.

4. Conclusions

Degradation testing with simulated river water was conducted with quaternary ammonium chlorides that contained alkoxymethyl or alkylmethyl substituents. The test showed that examined preparations were degraded at different periods of time. The calculated biodegradation rate constants indicate that the shortest degradation time was obtained for alkyl- and benzyl(alkoxymethyl)dimethylammonium chlorides. It was also found that biodegradation velocity decreased with increasing number of carbon atoms in the alkyl chain. The half-lives of the compounds were also estimated,and they ranged from 0.5 to 1.6 days depending on the type of QAS tested. Analysis of the data showed that all examined compounds were biodegradable in an aquatic environment. Based on the ^1H and ^{13}C NMR analysis of 1-decyl-3-cyklohexyloxymethyloimidazolium chloride, the hypothetical mechanism of QAS biodegradation was proposed. After biodegradation, only alcohols were present in the solution. The examined substances, their intermediates, and final biodegradation products did not cause genotoxic effects. These findings suggest that the presecnce of QAS compounds in natural bodies of water and thier consumption by humans will not create any carcinogenice health hazards.

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