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Water in cosmetics and *Caenorhabditis elegans* as an alternative model for lifespan assessment

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Abstract

Objective: Water, often considered a fundamental component of life, is the most commonly used ingredient in the formulation of dermocosmetic products, with waterless products being the exception. Dermocosmetic products can contain anywhere from 50% to 85% water, which contributes to their texture and specific characteristics. The chemical composition of water varies depending on its origin and can be categorized as highly mineralized or low mineralized. These compositions could impact the water's efficacy in anti-aging applications. In this study, the objective is evaluating the anti-aging properties of highly and low mineralized water with the model organism *Caenorhabditis elegans*.

Methods: In this article, we employed the alternative model organism *C. elegans* to assess the impact of 5 branded waters, one physiological water and one ultra-pure water on the model's lifespan, using the survival medium conventionally used for *C. elegans* as a comparison.

Results: Waters may have either a positive or a negative impact on the *C. elegans* lifespan expectancy. Our results indicate that only one of the water brands we assessed (Volvic®) had a significantly positive effect on worm longevity. In contrast, we found that two other brands (Hepar® and Contrex®) had a negative impact on the later stages of the worm's adulthood. Furthermore, we demonstrated that the impact of the brand water samples on lifespan expectancy varied depending on their physicochemical composition, in particular when ion concentrations were most extreme.

Conclusion: This study shows that the highly mineralized waters studied have a detrimental effect on the survival of *C. elegans*, and a preliminary test with ultra-pure water could not be completed due to its deleterious effect on the worms. This suggests the hypothesis that both highly mineralized and completely demineralized waters may not be the most suitable for skin formulations.

KEYWORDS

Caenorhabditis elegans, claim substantiation, formulation/stability, lifespan, safety testing, water

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RÉSUMÉ

OBJECTIF: L'eau, élément fondamental à la vie, est l'ingrédient le plus utilisé dans la formulation de produits dermocosmétiques, pour lesquels les produits sans eau restent des exceptions. Les produits dermocosmétiques contiennent 50% à 85% d'eau, contribuant à leurs textures et leurs caractéristiques spécifiques. La composition chimique de l'eau varie en fonction de son origine et peut être catégorisée comme hautement ou faiblement minéralisée. Cette composition peut impacter l'efficacité de l'eau pour ses applications anti-âge. Dans cette étude, l'objectif est d'évaluer les propriétés anti-âge d'eaux fortement ou faiblement minéralisées à l'aide de l'organisme modèle *Caenorhabditis elegans*.

METHODES: Dans ce travail, nous avons utilisé le modèle alternatif *C. elegans* pour étudier l'impact de 5 marques d'eaux, du sérum physiologique et de l'eau ultrapure sur ce modèle, en utilisant le milieu de culture standard de *C. elegans* comme contrôle.

RESULTATS: Les eaux peuvent avoir un impact positif ou négatif sur la longévité des *C. elegans*. Nos résultats indiquent que seule l'une des eaux étudiées (Volvic®) a un impact positif sur la longévité des nématodes. Au contraire, nous avons montré que 2 autres eaux (Hepar® and Contrex®) ont un impact négatif sur les derniers stades adultes. De plus, nous avons démontré que l'impact des eaux sur la longévité variait en fonction de leurs compositions physicochimiques, en particulier lorsque les concentrations ioniques sont extrêmes.

INTRODUCTION

Water is a common ingredient in cosmetics, the safety and quality of water is regulated by various organizations including in countries such as the US (Food and Drug Administration) [1], Canada [2], China (China Food and Drug Administration) [3], Japan (Japanese Pharmaceutical and Medical Device Act) [4], Australia (Australian regulatory guidelines for cosmetics) [5] and finally the European Union (the European Commission's Cosmetics Regulation) [6]. This last regulation sets out the requirements for the safety, quality and labelling of cosmetic products placed on the EU market, including those that contain water. In addition, the regulation sets limits on the concentration of certain substances in cosmetic products, including impurities and contaminants that may be present in water used in cosmetic formulations. For example, the regulation sets maximum limits for heavy metals such as lead and mercury, as well as for certain microorganisms that may be present in water.

The industry had to implement purification methods to stabilize the water quality in terms of composition to ensure good reproducibility of the manufacturing processes. There are various types of water used in the cosmetics industry, including:

- Untreated waters with distinct physicochemical compositions and specific characteristics:
 - Spring waters [7] come from underground water tables that are unpolluted, deep, or protected from human activity waste. Spring waters are naturally clean and safe for human consumption. There are around a hundred such sources in France.
 - Mineral water [7] is a category of water whose characteristics are defined by regulations. It must come from underground and have a stable chemical composition that does not require disinfection for consumption. Mineral water naturally contains levels of minerals and trace elements such as calcium, magnesium and potassium.
 - Thermal water [8] is a natural mineral water that has therapeutic properties recognized by the Academy of Medicine. Some of them have a dermatological orientation.
- Treated waters [9]:
 - Distilled water is one of the oldest and most commonly used technologies for treating water. It is purified through the process of distillation, which involves boiling water and collecting the resulting steam to remove impurities and contaminants.

- Deionized water permits its mineral ions, such as calcium, sodium and magnesium, removed through a process called ion exchange.
- Reverse osmosis water is more recent technology, widely used in the cosmetics industry. This water is purified by being forced through a membrane that filters out impurities, resulting in highly pure water.

Various technologies of varying degrees of sophistication are employed in the industry, with different processes utilized according to specific needs and available resources. It is worth noting, however, that cosmetic regulations do not require the use of high-purity water.

The wide variety of compositions of water used in cosmetic formulations could have an impact on skin ageing. Few studies have been conducted in this regard, and the purpose of this study, using an innovative alternative method, is to guide cosmetic formulators.

The experimentation with animals in the UE has been prohibited in several fields and for finished cosmetic products since 2004. In 2009, it has also been prohibited to market cosmetic products containing ingredients that have been tested on animals in the EU. For the most complex effects on human health, the entry into force of the ban on placing such products on the market took effect in 2013. New alternative methods to animal testing are consequently necessary. Initially proposed by Russell and Burch in 1959 [10], the new strategy of the “3 Rs” recently appeared in laboratories. The aim of this strategy is the reduction, refinement and replacement of laboratory use of animals [11].

Proposed in the 60's by Brenner [12], the *C. elegans* model can be used as an alternative model for toxicity testing [13]. This nematode has emerged as a model organism for phenotype-based drug screenings [14–16]. A study, made in 2011, validated a method to measure the *C. elegans* lifespan in 96-well microtitre plates and proposed a standardized protocol [17]. Meanwhile, the *C. elegans* is also an emerging model in biomedical and environmental toxicology [18].

Toxicity studies using mammalian models are believed to be both expensive and time-consuming. However, recent meta-analyses indicate that rodent models can predict specific toxic effects in humans in only 50% of cases [19–21].

Predictive toxicology tries to find alternative methods to improve effects prediction of extract, media or molecules for human health, by reducing the cost time needed for research development and the use of mammalian models tests.

Unlike in vitro testing, nematode toxicity assays provide data from a whole animal with a well-organized

digestive, reproductive, endocrine, sensorial and neuromuscular system. The *C. elegans* worm is about 1 mm in size. Moreover, 10s or 1000s of nematodes can be maintained in petri dishes or multi-well plates. Assessments for the effects of multiple compounds or mixtures at a wide range of concentrations can be carried out in a small space. The possibility to perform assays in a very small volume can be of great interest to laboratories. This is particularly the case when a solution, a media or a chemical to be tested is available in limited quantities because of chemical synthesis or natural availability.

Small *C. elegans* hermaphrodites can self-fertilize and produce roughly 300 progenies in 5 days. The life cycle of *C. elegans* is of 3 days at 20°C and millions of nematodes can be rapidly generated for experimental design. The *C. elegans* worm is a transparent organism which allows a direct visualization of internal structures without dissection. *Caenorhabditis elegans* is a recognized model for ageing research in laboratories worldwide [22]. Its cell lineage, cell positioning and neural networks have been totally mapped [23]. This is the first metazoan organism to have its genome completely sequenced [24]. The genome of *C. elegans* shares extensive homology to mammals. Many key cellular metabolic and signalling pathways are conserved between worms and humans. Studies designed to rank toxicity in *C. elegans* have shown a very good correlation with rodent oral LD₅₀ ranking [25].

The *C. elegans* model is used to determine the quality of water in the environment [26]. But, to date, no assessments about the potential impact of water composition on the *C. elegans* model have been done for cosmetic purposes.

An analysis of an electrolyzed reduced water (ERW) has been recently reported [27, 28]. When an electric current passes through water, it produces ERW near the cathode and electrolyzed-oxidized water near the anode. When assessed, the ERW significantly extended the nematode lifespan [27, 28]. It has been shown that the small amount of Pt nanoparticles contained in ERW can extend the longevity of the nematode by scavenging the reactive oxygen species also denominated ROS [28]. The free-radical theory of ageing, first suggested by Harman in 1956 [29] and developed since believed that ageing results from an accumulation of molecular damage caused by free radicals [30]. ERW is a powerful antioxidant. It can prevent oxidation and so extend lifespan. A report showed an insulin/IGF-1-like signalling-dependent effect of ERW for the lifespan extension of *C. elegans* [31]. The activity of the insulin/IGF-1-like signalling pathway can modulate to a great extent the *C. elegans* lifespan [32, 33].

The aim of this study was to investigate the impact of various brands of water (Hepar®, Contrex®, Cristaline®,

Evian® and Volvic®), as well as a physiological saline solution and ultra-pure water without mineralization, on the ageing phenomenon in the model organism *C. elegans*. These brands waters were chosen because they are readily available and have different chemical compositions. We compared the effects of these water sources to that of S-base, a commonly used culturing media for *C. elegans*.

MATERIALS AND METHODS

Materials

Seven different waters have been assessed: ultra-pure water (milliQ), physiological saline solution (NaCl 0.9%, Versol), Contrex® (Nestlé Waters), Hepar® (Nestlé Waters), Cristaline® Ste Cecile (Cristaline®), Volvic® (Danone Eaux France) and Evian® (Danone Eaux France). These waters were compared to the S-base Medium [34], the reference medium used in *C. elegans* ageing studies.

Bottles (50cL) of these commercial waters were bought directly in a shop and kept in a room at ambient temperature.

Mineral water and spring water are two common types of bottled water. Both natural mineral water and spring water must originate from a natural, protected and specific underground source and must be microbiologically safe to drink without treatment. But the natural mineral water must have a stable mineral composition. Evian®, Volvic®, Hepar® and Contrex® are mineral waters and Cristaline® is a spring water.

The chemical composition of these brand waters is described in Table 1.

TABLE 1 Ionic composition of studied waters.

mg/L	Contrex	Hepar	Cristaline Ste Cecile	Volvic	Evian	NaCl 0.9%	S-base
Calcium	468	549	39	12	80	0	0
Magnesium	74.5	119	25	8	26	0	0
Sodium	9.4	14.2	19	12	6.5	3540.6	2321
Potassium	2.8	4.1	1.5	6	1	0	1953.6
Silica	/	/	/	32	15	0	0
Bicarbonate	372	384	290	74	360	0	0
Sulfate	1121	1530	5	9	14	0	0
Chlorure	7.6	18.8	5	15	10	5459.4	3579
Nitrate	/	4.3	<1	7.3	3.8	0	0
Dry residue (180°C)	2078	2513	270	130	345	/	/
pH	7.4	7.2	7.7	7	7.2	7	6.5

Note: Ion content in mg/L of the brand waters used for experiments according to the supplier data.

Worm culture

Worms were maintained (Incubator Sanyo MIR-253) at 15°C and cultured at 20°C for experiments as described previously [12]. We used the wild type *C. elegans* strain: Bristol N2 and *Escherichia coli* HT115(DE3) strain as a food source. These strains were provided by the Caenorhabditis Genetic Center (funded by the NIH National Center for Research Resources).

Lifespan experiments

For each solution, 10 wells of an ELISA 96 plates were filled with five synchronized L4 larvae in a final volume of 200 µL of solution. Each worm-filled well was added with *Escherichia coli* HT115(DE3) strain. To preclude any influence of living bacteria on the experiments [35], the wells were filled with previously heat-killed HT115(DE3) bacteria. Nematodes were then allowed to grow and scored daily by direct observation under a dissecting microscope (Leica MZ6 – Leica Microsystems). Dead worms were scored every day for touch-provoked movement and individuals that displayed extruded internal organs as a result of possible injury during initial transfer for experiment preparation were included in statistical analysis as censored animals.

Internal controls for each experiment were done on S-base media as commonly used for *C. elegans* and described elsewhere [34]. To avoid offspring during the lifespan experiment, we added Fluorodeoxyuridine in each well as previously described [36]. The XLSTAT statistical software (Addinsoft) was used to plot survival data by the Kaplan–Meier method and the difference between survival curves

was calculated by using the LogRank test with 95% confidence. Lifespan experiments were repeated three times with identical results.

RESULTS

The *C. elegans* lifespan can be modified by waters

The Figure 1 brings together the mortality curves of control and waters treated worms. While the control condition shows a classical mortality curve for a *C. elegans* population at 20°C, we observed significant differences between the tested solutions. We decided to perform a more accurate analysis of the corresponding quantitative data. Table 2 summarize the total lifespan experiments performed. About 150 individuals were observed for each of the water assessed for a potential *C. elegans* lifespan impact.

Positive impact on longevity

In order to identify a beneficial water for worm longevity, we used successive pairwise comparison between all of the waters. As shown on Table 2, we were able to notice that the Volvic® water differs significantly from all of the others commercial waters assessed (Log-rank $p < 0.0001$). Furthermore, the Volvic® brand water appeared also to differ slightly but significantly from the control condition (Log-rank $p = 0.004$) and the physiological saline solution (Log-rank $p = 0.0002$). When we considered all of

the available quantitative data reported in Table 2, we also show that the Volvic® brand water displays the longer mean life span (15,78 days, SD=0.41) and the maximum expanded life span (30 days) in comparison with control (mean life span=13,46 days, SD=0.63; maximum life span=23 days). These results mean a positive impact of the Volvic® brand water on the life span expectancy of *C. elegans* as clearly shown in the Figure 2a where it is possible to notice a positive effect on the lifespan and, as unexpected, during the entire adulthood period.

No impact or negative impact on longevity

On the contrary, we were not able to detect the following solutions: Evian® (Figure 2b), NaCl 0.9% (Figure 2c), Cristaline® (Figure 2d), Hepar® (Figure 2e), Contrex® (Figure 2f), an extended life span effect (Table 2). Instead, the mean life span observed equalled roughly the one observed (Table 2) for the control (13,46 days, SD=0.63). In accordance with this observation, none of the previously listed waters differed significantly from the control by using the Log-rank test (Table 2).

However, when we compared the maximum life span observed with assessed waters, we noticed that a smaller life span was observed for Hepar® with 19 days.

These results reflect perhaps the cumulative effect of a drastic impact of the water on the *C. elegans* longevity during a prolonged period of adulthood and specially during the last part as it is clearly noticeable for the Evian® (Figure 2b), Hepar® (Figure 2e) and Contrex® (Figure 2f) brand waters.

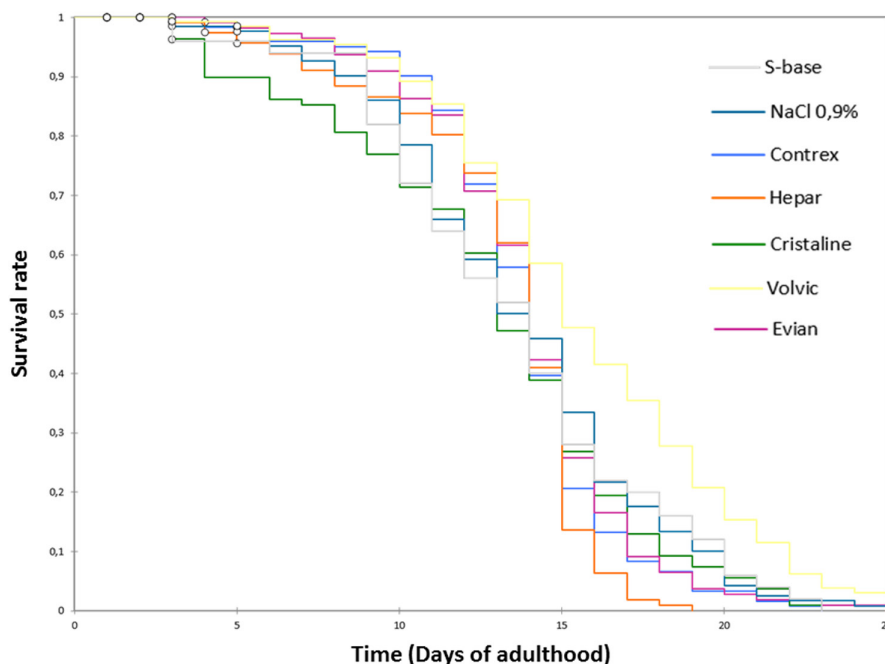


FIGURE 1 Waters can modify the *Caenorhabditis elegans* lifespan. The *C. elegans* lifespan analysis was performed at 20°C on ELISA 96 plates (see Material and Methods). See Table 1 for corresponding quantitative data.

TABLE 2 Water-dependent lifespan modulation of *Caenorhabditis elegans*.

Strain	Treatment	75th percentile	25th percentile	Mean life span days (\pm SD)	Max life span	Code	Statistics (KM analysis)	N (censored)
N2 (wt)	S-base	16	10	13,46 (0,63)	23	A	$p=0.004$ (B)	150 (22)
							$p=0.856$ (C)	
							$p=0.851$ (D)	
							$p=0.613$ (E)	
							$p=0.090$ (F)	
N2 (wt)	Volvic	19	13	15,78 (0,41)	30	B	$p=0.004$ (A)	147 (17)
							$p<0.0001$ (C)	
							$p=0.0002$ (D)	
							$p<0.0001$ (E)	
							$p<0.0001$ (F)	
N2 (wt)	Evian	15	12	13,91 (0,32)	26	C	$p=0.856$ (A)	151 (42)
							$p<0.0001$ (B)	
							$p=0.667$ (D)	
							$p=0.566$ (E)	
							$p=0.065$ (F)	
N2 (wt)	NaCl 0,9%	16	11	13,65 (0,38)	25	D	$p=0.851$ (A)	150 (30)
							$p=0.0002$ (B)	
							$p=0.667$ (C)	
							$p=0.406$ (E)	
							$p=0.018$ (F)	
N2 (wt)	Cristaline Ste Cecile	16	10	12,78 (0,46)	25	E	$p=0.613$ (A)	149 (41)
							$p<0.0001$ (B)	
							$p=0.566$ (C)	
							$p=0.406$ (D)	
							$p=0.268$ (F)	
N2 (wt)	Hepar	15	12	13,16 (0,30)	19	F	$p=0.090$ (A)	149 (39)
							$p<0.0001$ (B)	
							$p=0.065$ (C)	
							$p=0.018$ (D)	
							$p=0.268$ (E)	
N2 (wt)	Contrex	15	12	13,80 (0,28)	23	G	$p=0.630$ (A)	150 (29)
							$p<0.0001$ (B)	
							$p=0.577$ (C)	
							$p=0.345$ (D)	
							$p=0.941$ (E)	
							$p=0.212$ (F)	

Note: XLSTAT statistical software (Addinsoft, New York, NY, USA) was used to analyse survival data by the Kaplan–Meier method and differences between survival curves calculated using the Log-Rank test with 95% confidence. The third column represents the 75th percentile (the age when the survival fraction of animals reaches 0.25). The fourth column represents the 25th percentile (the age when the survival fraction of animals reaches 0.75). The fifth column presents mean lifespan of worms for each condition (in days). The sixth column shows maximum lifespan of worms for each water (in days). The eighth column represents statistics, p -value obtained. The last column shows total number of *C. elegans* scored and individuals censored during the experiments in brackets.

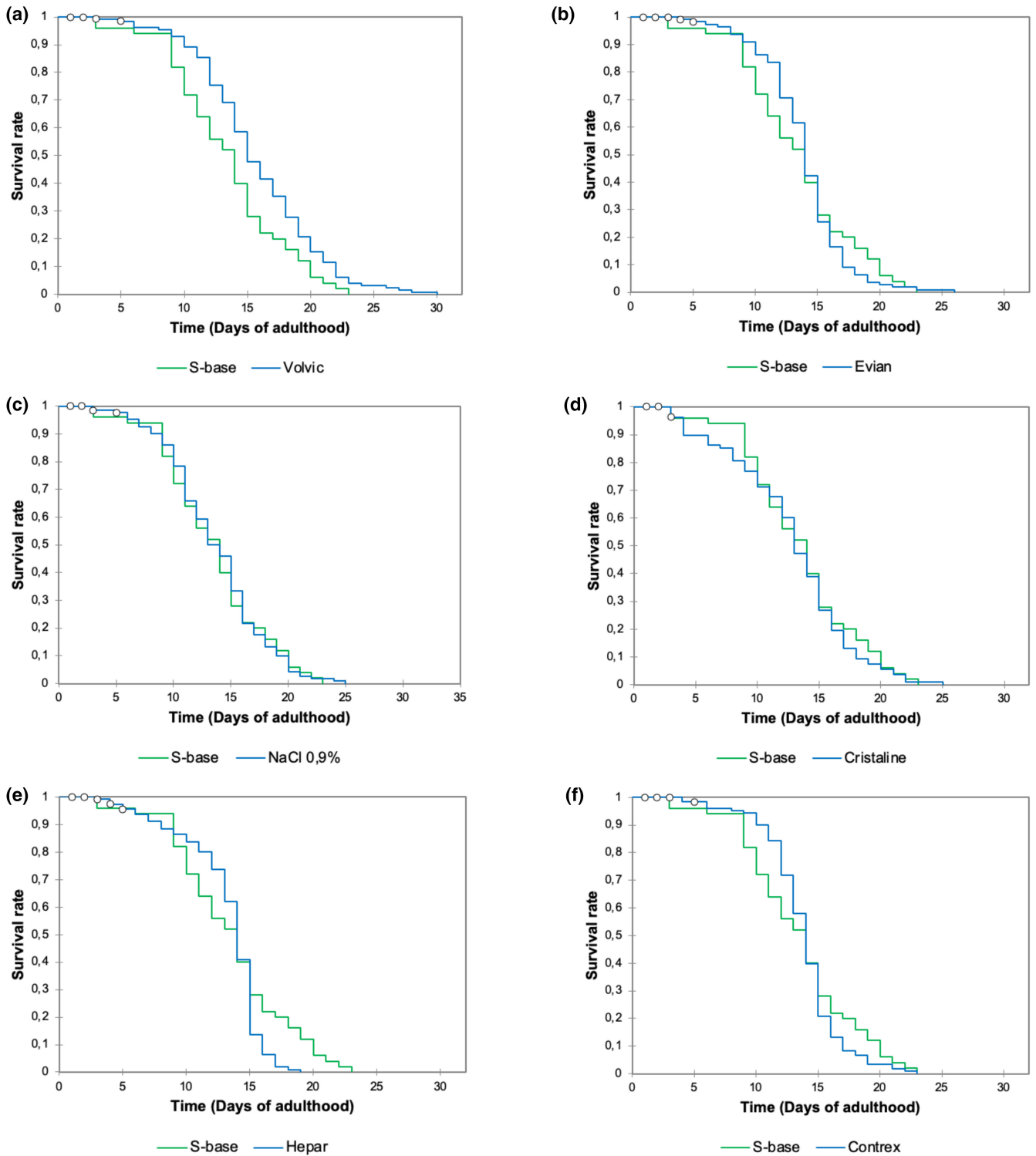


FIGURE 2 Impact of different waters on the *Caenorhabditis elegans* longevity in comparison with control. The *C. elegans* lifespan analysis was performed as for Figure 1 with the following waters: (a) Volvic®; (b) Evian®; (c) NaCl 0.9%; (d) Cristaline® Ste Cecile; (e) Hepar®, (f) Contrex®. See Table 1 for corresponding quantitative data.

When we analyzed the life span survival 75th percentile value (the age when the survival fraction of animals reaches 0.25), we noticed a difference of 1 day survival between the control (16 days) and both Evian® (15 days), Contrex® (15 days) and Hepar® (15 days) brand waters

(Table 2). In comparison, the 75th percentile value observed for the Volvic® brand water, which is beneficial for the worm longevity, was of 19 days (Table 2).

On the contrary, when we analyzed the life span survival 25th percentile value (the age when the survival

fraction of animals reaches 0.75), we noticed a difference of 2 days survival between the control (10 days) and these same waters (12 days) (Table 2).

For NaCl 0.9% (Figure 2c) and Cristaline® (Figure 2d) waters, no difference was observed with the control throughout the survival curve.

Regarding the ultra-pure water (Milli-Q) that was also tested, the results could not be reported as no curve could be plotted due to a strongly deleterious effect on the survival of *C. elegans*, which was quickly abbreviated. All the nematodes died after 3 days of experiment.

The brand water composition may explain the observed impact variations on the *C. elegans* longevity

The previous section shows that waters can modulate positively or not the life span expectancy of *C. elegans*. It is possible that the difference between the brand waters assessed here on the *C. elegans* ageing must rely on their physicochemical specificity.

The Table 1 presents the physicochemical composition of the waters used in this study.

Given in mind that the less beneficial brand waters for the *C. elegans* life span expectancy were Evian®, Contrex® and Hepar®, we can see that two of them show a very high level of sulfate ion in comparison with the others.

On the contrary, the more beneficial brand water identified in this study: Volvic® shows the lesser level of calcium, magnesium and bicarbonate. Contrastingly, even if the Cristaline® brand water shows the lesser level of sulfate, chloride, potassium and nitrate, its measured impact on longevity is null (Figure 2d; Table 2). These observations about a possible correlation between the physicochemical composition of the brand waters assessed here and their respective impact on *C. elegans* life span expectancy shed new lights about the difficulty to conclude simply on the basis of the higher content for given ions in a specific water. However, we can propose, as an alternative explanation, that a high content on sulfate seems well correlated with a lesser life span expectancy and shorter maximum life span as we noticed for both Hepar® (Figure 2e) and Contrex® (Figure 2f) brand waters. On the contrary, a possible equilibrium of the relative quantities between various ion species, as in the case for the Volvic® brand water, is perhaps the source of its positive impact on the *C. elegans* longevity during the entire adulthood. In fact, a positive effect on the longevity that spans the whole adulthood period of *C. elegans* is not common as expected and needs to be remarked.

DISCUSSION AND CONCLUSION

In the present study, we showed that waters may have either a positive or a negative impact on the *C. elegans* lifespan expectancy. By using *C. elegans* as a biomarker, previous reports succeeded in the detection on the impact of various stress sources such polluted water samples, heavy metals, gas as well as microwave induced stress [37–40]. However, these reports concerned acute stress and toxicity monitoring effects only. In this analysis, we monitored the impact of brand waters on a longer integrative stress variable like the lifespan expectancy.

Our results indicate that only one of the water brands we assessed (Volvic®) had a significantly positive effect on worm longevity. In contrast, we found that two other brands (Hepar® and Contrex®) had a negative impact on the later stages of the worm's adulthood. Furthermore, we demonstrated that the impact of the brand water samples on lifespan expectancy varied depending on their physicochemical composition, in particular, when ion concentrations are the most extreme.

It is interesting to note that the physiological solution (0.9% NaCl) as well as the natural spring water Cristaline® provide similar results to those obtained with S-base, the survival medium conventionally used for *C. elegans*.

The *C. elegans* model has been previously used for various toxicology assessments [13] and it is believed that the dose amounts needed for exogenous compounds to impact its physiology is likely the same as for humans [13]. This means perhaps that some of the water impacts on worm longevity here presented can be regarded with more attention even if not transposable directly to the human health.

The *C. elegans* ion homeostasis is highly regulated and numerous ion channels have been identified [41, 42]. The *C. elegans* ion channels are involved in many physiological processes like mechanosensory, locomotion, osmotic sensing, fluoride ion resistance, as well as development and reproduction.

As for example, it has been recently demonstrated that the knock-down of the mammalian Klotho gene homologues in *C. elegans* [43] can lead, as for mammalian, to a premature ageing syndrome [44]. Furthermore, the involvement of Klotho gene products has been shown to regulate the phosphate homeostasis of *C. elegans* as do in humans [45].

Therefore, while the impact of a particular water on *C. elegans* physiology may not directly translate to human health, it can provide a valuable tool for deciphering the molecular mechanisms involved in physiological responses through the available analyses provided by this alternative model.

This study shows that the highly mineralized waters studied have a detrimental effect on the survival of *C. elegans*, and a preliminary test with ultra-pure water could not be completed due to its deleterious effect on the worms. This suggests the hypothesis that both highly mineralized and completely demineralized waters may not be the most suitable for skin formulations. Further studies are currently underway to supplement this research and confirm the results using other mineral and spring waters.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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