Spectroscopic investigation of ionizing-radiation tolerance of a *Chlorophyceae* green micro-alga

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Abstract
Micro-organisms living in extreme environments are captivating in the peculiar survival
processes they have developed. *Deinococcus radiodurans* is probably the most famous
radio-resistant bacteria. Similarly, a specific ecosystem has grown in a research reactor storage
pool, and has selected organisms which may sustain radiative stress. An original green
micro-alga which was never studied for its high tolerance to radiations has been isolated. It is
the only autotrophic eukaryote that develops in this pool, although contamination possibilities
coming from outside are not unusual. Studying what could explain this irradiation tolerance is
consequently very interesting. An integrative study of the effects of irradiation on the
micro-algae physiology, metabolism, internal dynamics, and genomics was initiated. In the
work presented here, micro-algae were stressed with irradiation doses up to 20 kGy (2 Mrad),
and studied by means of nuclear magnetic resonance, looking for modifications in the
metabolism, and on the IN13 neutron backscattering instrument at the ILL, looking for both
dynamics and structural macromolecular changes in the cells.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Life is everywhere, including extreme environments on Earth. The extremophile classes [1] include for instance organisms surviving high salt concentrations (*Haloarcula* sp.) [2, 3], toxic metals and high pressures (*Bacillus infernus* and other obligate barophiles) [4], acidity (*Thiobacillus* sp.) [5], low (*Aquaspirillum arcticum*) [6, 7] or high temperatures (*Aquifex pyrofilus*) [6], vacuum (*Bacillus subtilis*), and desiccation (many bacteria). Additionally, the *Deinococcus radiodurans* bacterium has been found to exhibit an extraordinary ability to survive ionizing irradiation inducing DNA damage [8]. The archeon *P. abyssi* and the cyanobacteria *Chroococcidiopsis* sp. [9] may also sustain ionizing radiations, but to a lesser extent, as shown in table 1.

Ionizing-irradiation resistance is usually considered to be related to desiccation tolerance [9, 10], as they both induce extensive DNA damage with double-strand breaks. A recent study on *D. radiodurans* has described in detail an original two-stage DNA repair mechanism called extended synthesis-dependent strand annealing [10]. This process requires at least two copies of the genome, as in *D. radiodurans* [8, 10] and *Chroococcidiopsis* sp. [9].

In this study, we report on an ionizing-irradiation tolerant micro-alga. This green micro-alga, belonging to the *Chlorophyceae* class, was discovered in the water cooling systems and in the nuclear waste fuel elements storage pool of a research reactor. It is the only autotrophic eukaryotic organism to grow in such medium, although contamination possibilities
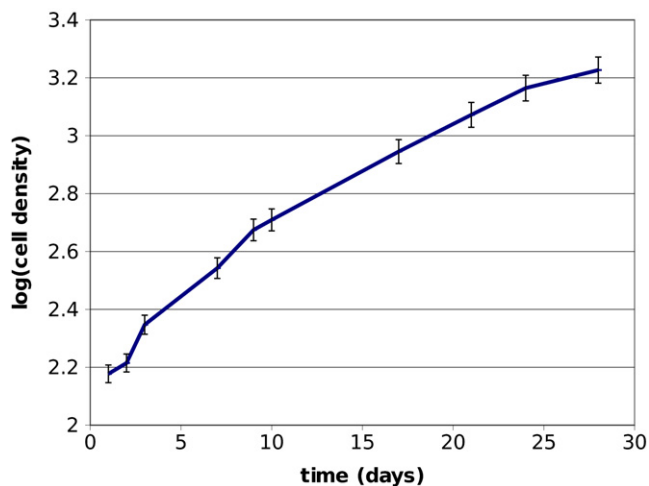
Figure 1. Growth rate of micro-algae. The line is a guide to the eye.

Table 1. Ionizing-irradiation resistance level (shoulder of ionizing-irradiation tolerance) for organisms sustaining high doses. Exact tolerance varies depending on the strain.

Genus	γ-irradiation resistance
Chroococcidiopsis sp. (cyanobacteria)	2.5 kGy
Pyrococcus abyssi (archeae)	3 kGy
Deinococcus radiodurans (bacteria)	6–15 kGy

from outside environment are not unusual. This adaptation indicates an irradiation tolerance capacity higher than that of other eukaryotes. The study of this micro-alga is consequently of peculiar interest, all the more so as there is little knowledge on algae resistant to gamma irradiation.

An integrative study of the effects of irradiation on this micro-alga physiology, metabolism, internal dynamics, and genomics was initiated. In the work presented here, micro-algae were stressed with irradiation doses up to 20 kGy (2 Mrad), their survival was assessed as a function of the irradiation dose, and changes in metabolism, internal dynamics and structure brought by irradiation were studied respectively using nuclear magnetic resonance (NMR) and neutron backscattering on the IN13 instrument at the ILL.

2. Extraction of the micro-algae

The light water (H_2O) swimming pool of the research reactor is populated with two micro-organisms: a green micro-alga and a saprophyte fungus. The swimming pool is filled with high purity deionized water which serves as cooling media for the waste nuclear fuel elements. The conductivity is kept very low by means of filters and ion-exchange resins. The organisms may survive in this very poor nutritive media using the UV light from surrounding neon tubes, dust deposited on the water surface, and dissolved CO_2.

Even though the radioactivity level in the water may vary from 30 μGy h^{-1} to more than 300 Gy h^{-1} in a few places,
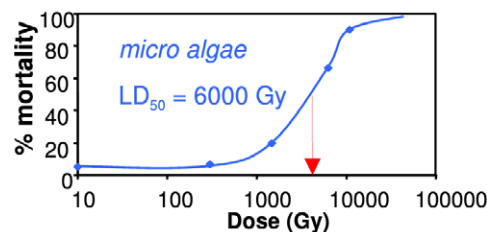

Figure 2. Micro-algae mortality versus radiation dose. The line is a guide to the eye.

the mean activity on the algae colonies in the swimming pool is around 150 μGy h^{-1} (measured using a BEFIC IF 103 dosimeter, 1 Gy = 1 J kg^{-1} = 0.1 krad). This ecosystem has been stable for at least 15 years.

The micro-algae cells are typically 7 μm $\times$ 4 μm, elliptically shaped. The population doubling rate is about six days (see figure 1). In this respect, the metabolism of the micro-algae is much slower than those of other radio-resistant organisms such as *D. radiodurans*. Moreover, like some other extremophiles, the micro-algae is desiccation tolerant, may survive in acid solutions (pH = 2.5), ultrasound cleaning, solvents (acetone, alcohol), and recover from freezing and complete darkness.

Algae were harvested from a flat surface on an immersed light 2 m below the water surface of the reactor swimming pool. They were cultured aerobically in order to obtain larger cell quantities. As the growing rate is limited, the culture required about a year for 10 g of cells to be obtained for NMR and neutron spectroscopy measurements.

3. Irradiation of cells

Micro-algae samples were deposited on top of waste fuel elements, integrating an irradiation rate of 330 Gy h^{-1}. The highest dose (20 kGy) was integrated from a closer location next to the fuel element, with a rate of 2000 Gy h^{-1}. These rates were estimated from the deactivation calibration of waste fuel elements measuring the activity as a function of distance and time after the end of the reactor cycle [11]. As a comparison, the mean irradiation dose integrated by plants after the Chernobyl accident was estimated to a few hundreds of grays [12].

During the irradiation, each sample was inserted in a 14 ml 17 mm $\times$ 100 mm Falcon PPN tube, all stored in an aluminum container which was positioned on the top of a fuel element immersed in the reactor swimming pool. The ionizing irradiation is mainly constituted with broad energy spectra γ-rays and fast neutrons.

Aliquots of each sample were extracted immediately after irradiation in order to count the surviving cell density, using a Malassez plate and a 1% neutral red dye. Optical numbering was performed on 40 grid cells on the plate. The irradiation survival rates of micro-algae, shown in figure 2, were measured as about 80%, 33%, and below 20% for 1.5, 6 and 10.8 kGy ionizing radiations respectively, with an uncertainty of 5%.
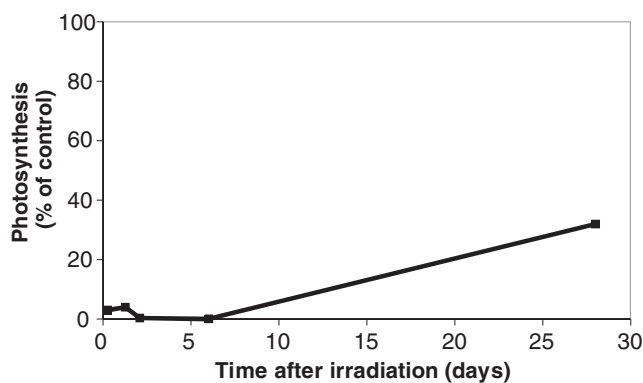
Figure 3. Recovery of micro-algae photosynthesis over time after a 20 kGy irradiation. The line is a guide to the eye.

The irradiation dose leading to the immediate mortality of half of the population (LD_{50}) is about 6 kGy.

After exposure to a high irradiation dose (2 kGy h^{-1} for 5–10 h), micro-algae colonize the culture medium again within a few weeks. Figure 3 shows the recovery of photosynthesis in a sample irradiated at 20 kGy. Photosynthesis was measured through O_2 emission of illuminated micro-algae. O_2 emission was monitored polarographically using a Clark-type oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) at 20 °C, in a 1 ml illuminated cell. Photosynthesis is an indicator of the physiological state of the cells. It was measured immediately after irradiation and dropped nearly to zero. However, it recovered to about a third of the control value after four weeks (see figure 3).

Even though *D. radiodurans* is undoubtedly more ionizing-irradiation resistant than this micro-alga, this latter is, to our knowledge, one of the few eukaryotic algae found to resist such high doses.

4. Effect of radiation on metabolism (NMR study)

After irradiation, modification in the micro-algae metabolism was assessed by comparing metabolic profiles of control and irradiated cells. Metabolites define the biochemical phenotype of the organism or the cell. Their qualitative and quantitative determination informs of the biochemical status of the organism, cellular functioning, and pathways affected by stress or disease.

Metabolic analysis of biological materials is performed using different techniques including powerful identification techniques such as mass spectrometry [13] or NMR [14], separative techniques such as gas chromatography [15], liquid chromatography [16], and capillary electrophoresis [17], or a combination of separative and identification techniques. Most investigations are based on NMR and mass spectrometry. NMR is non-intrusive, rapid and selective. Compared to ^{13}C NMR, ^{1}H NMR is more sensitive. In ^{1}H NMR, one compound gives rise to many signals, due to multiple spin–spin interactions between protons and other nuclei. Despite the complexity of the spectra, a large number of metabolites could be identified and quantified using this technique.

Micro-algae samples were irradiated at 450, 2000, and 20 000 Gy. Prior to ^{1}HNMR analysis, metabolites were extracted from the control and the irradiated cells using an aqueous acid solution as follows. Fresh samples were centrifuged at 4 °C at 2800*g* for 3 min; the pellet was frozen in liquid nitrogen and ground to powder with 70% perchloric acid (0.1 ml g^{-1} wet weight). Maleate (2.5 μmol g^{-1} wet weight) was added as internal standard. The powder was defrosted at 0 °C and centrifuged at 4 °C and 15 000*g* for 10 min to remove particulate matter and proteins. The supernatant was neutralized with 2 mol l^{-1} KHCO$_3$ to pH 5.2 and centrifuged at 4 °C to remove the KClO$_4$ precipitate. The resulting supernatant was frozen in liquid nitrogen, lyophilized, and dissolved in 0.5 ml D$_2$O. After pD adjustment to 7.00 ± 0.05 with DCl and NaOD and addition of 0.125 μmol (trimethyl)propionate-2,2,3,3,-D$_4$, samples were lyophilized again and finally dissolved in 0.5 ml D$_2$O.

The ^{1}H NMR spectra were recorded in D$_2$O at 25 °C using a 400 MHz Bruker (Wissembourg, France) spectrometer equipped with a 5 mm direct probe. The free induction decay resolution was 0.13 Hz, the pulse angle was 60° and the repetition period was 3.82 s for each of the 512 transients. Free induction decays were Fourier transformed with 0.3 Hz line broadening, phased and baseline corrected. The resulting spectra were aligned by shifting (trimethyl)propionate-2,2,3,3,-D$_4$ signal to zero. Metabolites were identified on the spectra by their chemical shift and by spiking the sample with authentic compounds. Peak areas were integrated using the WIN-NMR software (Bruker Biospin, Karlsruhe, Germany) after local baseline flattering around each integration region. The areas, related to that of the internal standard, provided the concentration of each metabolite in the sample. The 0, 2, and 20 kGy ^{1}H NMR spectra are shown in figure 4.

The concentration of various sugars (saccharose, trehalose, and glucose), amino acids (glutamate, alanine, valine, leucine, and isoleucine) and organic acids (malate and succinate) has been studied as a function of integrated dose for 0, 450, 2000 and 20 000 Gy (see figure 5).

Irradiation triggered an increase in some amino acid pools (alanine, valine, leucine, isoleucine) in the micro-algae at low doses that did not induce immediate mortality (450 Gy), suggesting an action on proteins with amino acid release like that observed in carbohydrate stresses [18]. Most metabolite concentration, including other amino acids, organic acids, and sugars, was affected from a 2 kGy dose. At very high irradiation doses, most metabolic pathways stopped functioning, leading to the disappearance of most of the metabolites.

5. Effect of irradiation on structure (neutron scattering study)

Irradiated samples originating from the same alga strain were studied *in vivo* using the IN13 neutron backscattering spectrometer at the Institut Laue-Langevin[5] for measuring
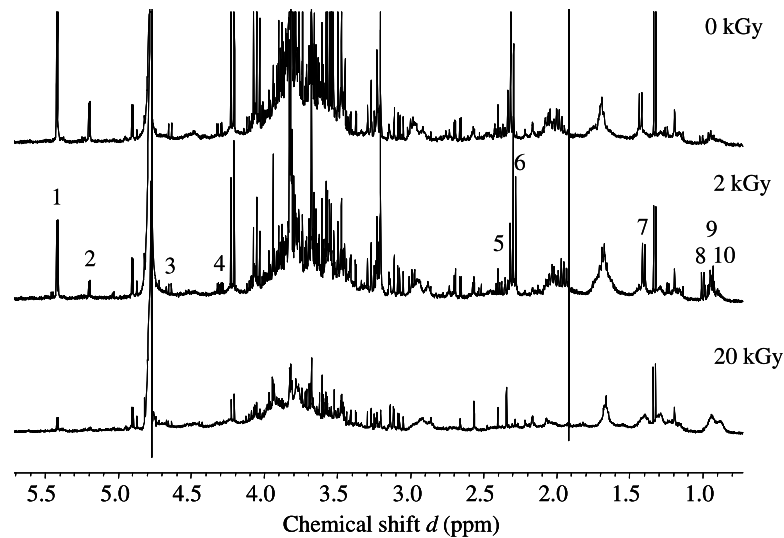
Figure 4. ^{1}H NMR spectra of micro-algae after 0, 2 and 20 kGy irradiation. 1: saccharose, 2: trehalose, 3: glucose, 4: malate, 5: succinate, 6: glutamate, 7: alanine, 8: valine, 9: leucine, 10: isoleucine.
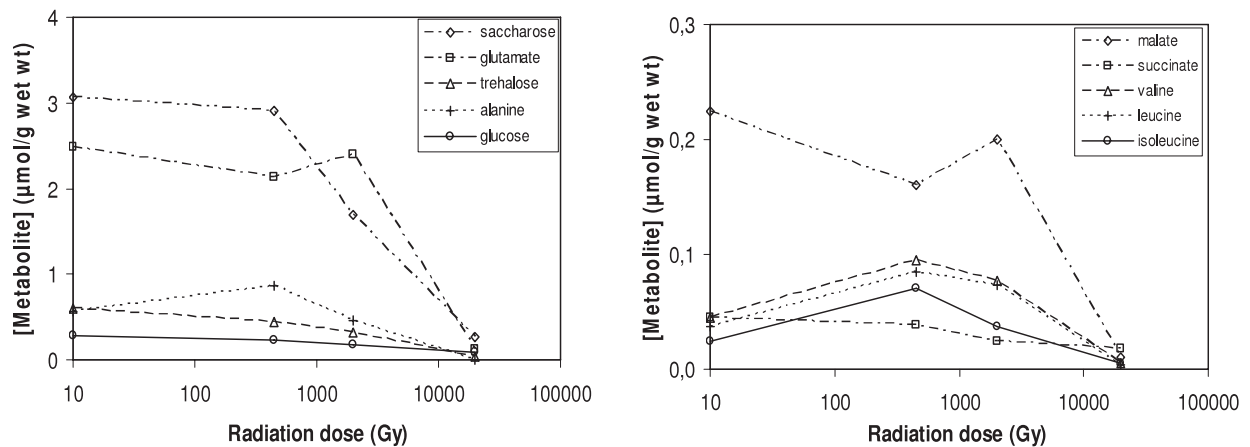

Figure 5. Change in metabolite content as a function of the radiation dose. The lines are guides to the eye.

the mean macromolecular displacement and possible changes in the cell dynamics. This spectrometer is designed to study elastic (structure) and inelastic (dynamics) changes in biological systems, with a high energy resolution and high momentum transfer, mostly from incoherent scattering. The spectrometer was used with an incoming neutron wavelength of $\lambda_i = 2.23$ Å. The resulting energy transfer resolution was of $\delta E = 8$ μeV in the accessible energy and momentum transfer dynamical range -0.1 meV $< \omega < +0.1$ meV and 0.3 Å $< q < 5.5$ Å^{-1}. In time and space, the instrument may thus record structure and motions for lengths 1.2 Å $< r < 21$ Å (i.e. include the size of protein side chains and amino acids) and times 6 ps $< t < 700$ ps.

After irradiation, algae samples were washed 3 times with 5 ml D_2O and centrifuged at $2800g$ for 3 min, and finally at $3500g$ for 5 min, to remove intercellular H_2O. Samples of about 1.1 g were obtained as a dark green paste. It was checked this D_2O treatment did not disturb cell physiology, as respiration and photosynthesis after 24 h were identical to those of samples maintained in H_2O.

Samples were inserted into an AG3Net aluminum plate sample cell of thickness $e = 1$ mm. The neutron transmission was about 90%. By means of an ILL closed-cycle refrigerator, temperature was kept constant at $T = 280$ K during the neutron scattering quasi-elastic measurements (dynamics), and was scanned from 280 K up to 310 K for the elastic (structure) study. The only parameter that was varied between different samples was the irradiation dose.

The scattering intensity is recorded as a function of the energy transfer ω and momentum transfer q, for each sample, γ-irradiation dose and temperature. The signal was corrected from the background and the empty sample cell contributions, and normalized to the vanadium scattering in order to account for the detector efficiency. As the samples contain essentially protonated macromolecules surrounded by water, we may assume that most of the scattering is incoherent. Additionally, the algae cells may be considered as a highly disordered gel, for which the coherent contribution will be negligible. Neutrons are highly sensitive to protons, so that the neutron scattering intensity will record in practice the contributions of side chains
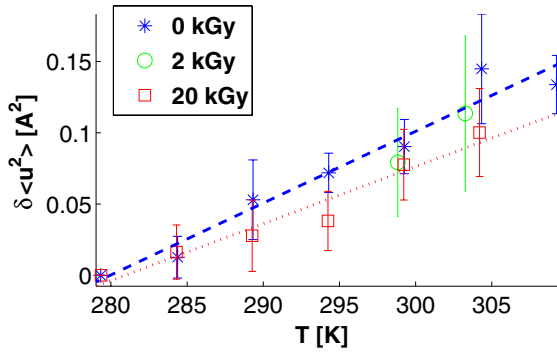
Figure 6. Micro-algae mean square displacement temperature shift w.r.t. $T = 280$ K versus integrated dose. Lines are linear fits to the curves, in order to extract the associated resilience k. The $\langle u^2 \rangle$ values at $T = 280$ K are around 1.4 Å^2.

and backbone to which they are linked, as well as the remaining water.

Let us consider one atom of a large macromolecule in the alga cell. Its movement, constrained by the local environment, may be modeled by a potential energy ΔU over a typical cage length Δl. The associated force $F = -\Delta U/\Delta l$ maintains the atom in the potential well, and corresponds, in the harmonic approximation, to a spring force constant k so that $\Delta U = 1/2\, k(\Delta l)^2$ [19, 20]. In such a complex biological system as an *in vivo* algae cell measurement, these quantities should be averaged over the whole cell and the measurement space and time window. In these systems, we denote this space and time average of the squared atom movement length scale Δl^2 as the mean square displacement $\langle u^2 \rangle$. Following Bicout and Zaccai [19, 21], the neutron elastic incoherent scattering intensity may then be written, under the condition $\langle u^2 \rangle q^2 \sim 2$, as $I_{inc}(q, \omega = 0) = I_0 \exp[-q^2 \langle u^2 \rangle/6]$ where I_0 is a constant. The slope of $\ln(I_{inc})$ as a function of q^2 in the range 1.2 Å$^{-1} < q < 2.2$ Å^{-1} gives an estimate of $\langle u^2 \rangle$ in the time and space ranges measurable by the instrument, for each temperature and irradiation dose. Within this framework, $\langle u^2 \rangle$ may be considered as a measure of the mean displacement of macromolecules in large conformational cages.

We have plotted in figure 6 the mean square displacement temperature shift $\partial \langle u^2 \rangle$ for the control (0 Gy dose), 2 and 20 kGy doses, with regard to $T = 280$ K. Bicout and Zaccai [19] have shown that this quantity is related to an effective resilience force constant $k = 0.00276/(d\langle u^2 \rangle/dT)$. Using a linear fit with the Marquardt–Levenberg optimization algorithm to the data points, we have then derived the resilience k values 0.54 ± 0.05 N m^{-1} and 0.69 ± 0.11 N m^{-1} for the control and the 20 kGy irradiated samples respectively. In the present study, the observed resilience limited increase can only be related to the only parameter that was changed between measurements: the irradiation dose. All other parameters such as the solvent composition and the cells strain were identical. The error bar on the mean square displacement $\langle u^2 \rangle$ (see figure 6) and the uncertainty on the resilience k arise from the limited signal to noise ratio as the IN13 spectrometer is a low flux instrument.

In longer measurements, we acquired the quasi-elastic intensity in order to estimate changes in the cell dynamics with irradiation. However, the IN13 spectrometer is severely flux limited, which prevented identifying any change in the frequency distributions of movements in the measurement range.

6. Discussion and conclusions

Metabolism changes investigated using ^{1}H NMR show that sugar concentrations remain nearly constant (or decrease a little) and then lower slowly with increasing irradiation dose, which may indicate that the sugar energy source pathways are affected but still maintained. Moreover, the pools of some free amino acids increase in the cell, even at low irradiation doses that do not induce immediate mortality. These variations probably go together with several phenomena, among which is damage to DNA.

One point is that repair mechanisms require a substantial energy to function. Once the available intracellular carbohydrates have been burnt, cells may use directly broken protein fragments and slowly degrade their own content via macro-autophagy [18, 22] in order to feed the mitochondria with oxidizable materials for ATP production. The consumption of carbohydrates, used as cellular energy sources under normal living conditions actually increases during stresses. However, as carbohydrates are compartmentalized in plant cells, an increase in energy needs will deplete the cytosolic carbohydrate pool. Meanwhile, carbohydrates contained in plastid as starch will be hydrolyzed and supply mitochondria with substrate, but very slowly. Moreover, the big vacuolar sugar stocks will not be exported from the vacuole very quickly. This results in the metabolite variations observed by means of NMR. On one hand, the overall carbohydrate pool in the micro-algae will not change drastically. On the other hand, the increasing sugar consumption will result in a sugar starvation; the concentration of available sugar being insufficient for normal respiration. This may be solved by autophagy reactions which will provide respiration with substrates. Protein lyses, which result in an increase in amino acids, are examples of autophagies. The increase in valine, leucine, and isoleucine may result from the autophagy of membrane proteins (in which these amino acids are among the main residues) [18]. These processes are reversible and do not lead to cell death.

Another point is that the repair of DNA damage induced by irradiation also requires amino acids. Increasing amino acids amounts may be synthesized during a repair mechanism taking place in cells. Currently, not less than seven DNA repair mechanisms have been envisaged to take part in the re-assembly of DNA fragments caused by radiative double-strand breaks [10]. The most efficient mechanism, from which *D. radiodurans* gains its extreme resistance, requires genome copies (at least $4n$) [8–10]. Even though algae chloroplasts create typically hundreds of copies of their plastidial genome [23], the nuclear genome might rather be unique (only $2n$) in the micro-algae studied here, and the latter repair mechanism may not apply.

In the single-nuclear-DNA hypothesis, the consequence of the DNA damage for the micro-algae is probably less than that for *D. radiodurans*, especially as the replication rate of the micro-algae is slow and few cells are dividing at a time.

The increase in amino acid concentration in the cell may thus be either directly related to the DNA repair mechanism, or may be part of the energy production pathway under stress.

Lastly, amino acid pools may also increase as a degradation of proteins occurs following the direct physical effect of ionizing radiation. Gamma irradiation in biological systems induces highly excited electronic states in water and macromolecules such as proteins and DNA, producing free radicals like reactive oxygen species, breaking proteins, causing DNA single- and double-strand breaks, and generating damaged bases. As regards proteins, free radicals, such as protein hydroperoxides, finally decay into hydroxide residues, in particular valine and leucine hydroxides [24]. Protein turnover will increase as non-functional enzymes and fragments will then be destroyed by hydrolases into amino acids and be reused for protein translation, this phenomenon being observed at sub-lethal irradiation dose.

We showed that neutron backscattering was suited to *in vivo* studies of biological material. However, elastic scattering studies are usually performed on crystalline (monocrystals and powders) or monodisperse (polymers, glasses, and liquids) samples. Here we analyzed living biological samples. The micro-algae samples were homogeneous as these algae are unicellular and all the cells were analyzed at the same growth stage. Measurement conditions did not disturb the physiological state of the cells, and measurement times (typically 10 h) are far below the characteristic living times of these algae, which minimized ageing of the sample and ensured stable physiological parameters for the population during measurement.

The total mean square displacement of atomic motions was assessed and its dependence on temperature was evaluated on the IN13 instrument. The force constant k thus obtained, which represents the macromolecular resilience of the algae sample, was 0.54 ± 0.05 N m^{-1} in control algae. This value is of the same order as that previously measured on IN13 where the resilience for extremophile bacteria living at 4, 75, and 85 °C was found as 0.21 ± 0.03, 0.67 ± 0.11, and 0.60 ± 0.01 N m^{-1}, respectively [6]. In the present study, the micro-algae metabolism has adapted to an irradiative stress and the resilience of the control algae is already significantly high, comparable with those of *T. thermophylus* and *A. pyrofilus*.

Under additional irradiation, the resilience of micro-algae increases even more, up to $k = 0.69 \pm 0.11$ N m^{-1}. A radiative stress does not produce the same effects as a thermal stress; nonetheless, the neutron backscattering results for micro-algae according to the irradiation dose may be compared to those for extremophile bacteria subjected to a thermal stress. In these organisms, temperatures higher than their usual living temperatures lead to protein denaturation, and the associated resilience then drops significantly [6]. Apparently the effect of a high irradiation on micro-algae does not lead to protein denaturation, but rather reduces the flexibility of macromolecules measured by the IN13

spectrometer. The resilience does indeed provide information on the stiffness of cellular molecules in their environment. In this framework, the observed increase under irradiation suggests that the neutralization of major biochemical pathways tends to reduce the mean conformational cage extension. To our knowledge, the results reported here are the first neutron elastic scattering studies performed on such complex organisms that are eukaryotic micro-algae.

In conclusion, a *Chlorophyceae* green micro-alga has been found to resist high γ-irradiation doses, up to 6 kGy. Even higher doses induce an appreciable decrease in the survival rate, but the micro-algae colonies may still recover after a month. Even though the associated tolerance mechanism is not known today, we have performed spectroscopic investigations which provide a few macroscopic indicators for the cell behavior during irradiation. Most probably, the intracellular amino acid release is a sign of an intense DNA repair activity, as the sustained sugar concentration suggests.

Acknowledgments

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