# ARTÍCULO

## Microalgae: the first nuclear engineers?

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## ABSTRACT

Self-sustaining nuclear chain reactions ran spontaneously 1.7 billion years ago at Oklo (Gabon, Africa) are a mystery. It was hypothesized that the microalgae concentrated enough rich-uranium in Oklo as for a natural reactor to start operating. The key to understanding as microalgae could do this is in an extremely U-contaminated pond of Saelices uranium-mine (Spain). Some microalgae colonized this extreme pond due to spontaneous mutations of single-genes. These U-resistant microalgae concentrate 115 mg U/g dried-biomass by bio-adsorption and bioaccumulation and are able to enrich uranium producing isotopic fractionation $^{235}U/^{238}U$. Consequently, microalgae could be able to build a nuclear reactor in appropriate circumstances.

**Keywords:** Microalgae; Uranium; Nuclear reactors.

## RESUMEN

*Microalgas: ¿los primeros ingenieros nucleares?*

Las reacciones nucleares en cadena auto-sostenibles que ocurrieron espontáneamente hace 1.700 millones años en Oklo (Gabón, África) son un misterio. Hipotéticamente las microalgas concentraron suficiente uranio enriquecido para que un reactor nuclear natural comenzara a operar. La clave está en un estanque contaminado por uranio en la mina de Saelices (España). Algunas microalgas colonizaron este estanque extremo debido a mutaciones espontáneas de genes individuales. Estas microalgas Uranio-resistentes concentran 115 mg U / g de biomasa seca mediante bio-absorción y bioacumulación, siendo capaces de enriquecer uranio produciendo fraccionamiento isotópico $^{235}U/^{238}U$. Estas microalgas podrían construir un reactor nuclear en circunstancias apropiadas.

**Palabras clave:** Microalgas; Biorremediación de uranio; Reactor nuclear.

## 1. INTRODUCTION

When on 2nd December of 1942 Enrico Fermi started the nuclear reactor Chicago Pile-1 (CP-1) as part of the Manhattan Project at the Metallurgical Laboratory of the Chicago University, he was convinced that CP-1 was the first nuclear fission reactor running on Earth, he was far removed from reality. 1.7 billion years ago, at Oklo in Gabon, Africa, 16 natural nuclear fission reactors (i.e. a uranium-rich deposit where self-sustaining nuclear chain reactions have occurred) took place and ran approximately for hundred thousand years (1-2). Oklo was discovered in 1972 by the French physicist Francis Perrin while he was analysing isotope ratios, a possibility previously predicted by Kuroda (3).

A nuclear chain reaction took place in a uranium-rich deposit inundated with water (that acted as a neutron moderator) at Oklo originating a natural nuclear reactor (1, 4-5). The key factor for this event to happen was that the fissile isotope $^{235}U$ reaches around 3.1% of the total uranium amount, a similar amount as the used in some of human manufactured reactors.

Lovelock (6) proposed that the microalgae could have concentrated $^{235}U$ in Oklo. But to date no experimental evidences were provided in this regard.

For become able to build a natural nuclear reactor microalgae need to meet three characteristics:

i) being able to survive in an environment contaminated by uranium,

ii) being able to concentrate uranium,

iii) being able to produce isotopic fractionation enriching the relationship $^{235}U/^{238}U$.

It seems unlikely that these three characteristics take place simultaneously in microalgae. First, surviving under uranium contamination environment is not straightforward. Uranium is a hazardous element owing to its toxicity as heavy metal as well as its radioactivity (7). Second, neither seems simple that microalgae bio-accumulate uranium because it is a material that has no biological utility. But the hardest challenge to meet is that microalgae could get isotopic fractionation of uranium.

How microalgae could acquire these 3 qualities?

Astonishingly, there are experimental evidences that some microalgae species were able to do this in a pond extremely contaminated by uranium at the Saelices U-mine (Salamanca province, Spain). And they have recently started: since the sixties of past century.

In this paper we review these surprising evidences.

## 2. AN EXTREME ENVIRONMENT: THE URANIUM-POLLUTED POND AT SAELICES MINE.

Huge uranium deposits occur in the fracture areas in shale and schist of the pre-Ordovician schist-greywacke complex that forms part of the paleozoic basement of the Hesperian Massif in Iberian Peninsula (8). As anecdotic historian, the uranium mining of the Hesperian Massif was an important radio supply source for the experiments conducted by Madame Curie. The most important of these uranium deposits with a total volume of 25 million cubic meters, and an ore grade ranging from 400 to 800 mg $kg^{-1}$ of uranium are in Saelices, Salamanca (Spain) which were exploited in mining since 1960 until 2000. As a result of these mining activities of static and dynamic acid lixiviation there are around 30 ha of uranium-polluted ponds containing 1 million $m^3$ of uranium-polluted water (Figure 1).

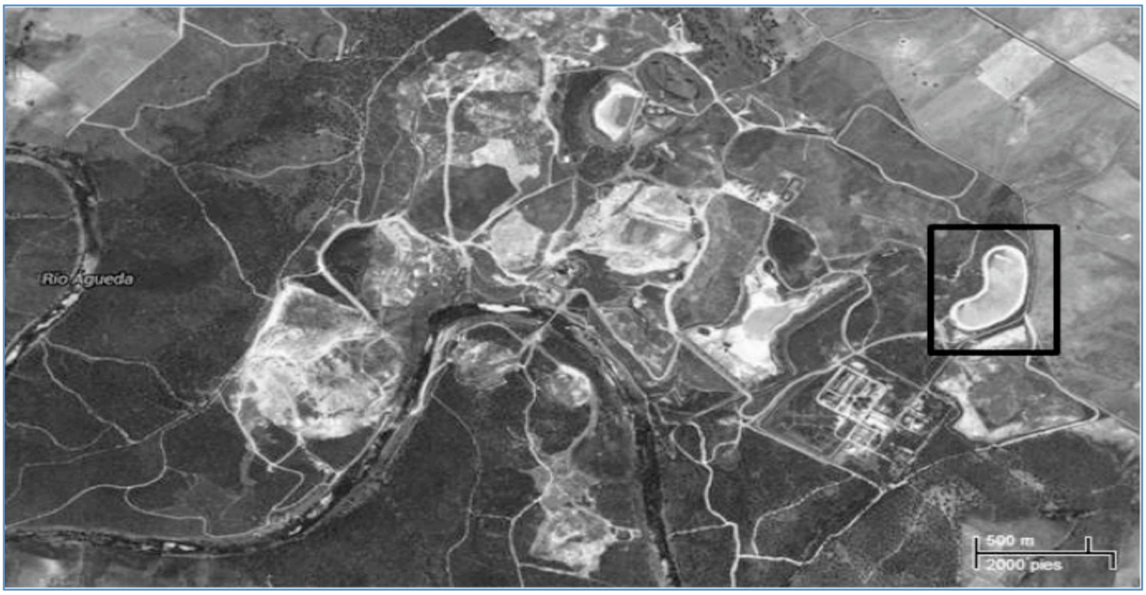


**Figure 1.- Air image of Saelices mine sampled area, located in the Saelices el Chico municipality, in Salamanca, Spain.** The square indicates the mining evaporation pond from where the water samples were taken.

We studied a huge evaporation pond at the Saelices mining area from March 2012. This pond is an extreme ecosystem with uranium contamination, pollution by other heavy metals, acidity and radioactivity as shows in Table 1. Median value of uranium concentration obtained during a yearly cycle exceeds 833 times the uranium levels in water considered safe by the EPA. We also studied a natural pond at Zamarra, without mining activity that can serve as a baseline of pollution levels in that area before human activity (Table 1).

**Table 1.**- Environmental conditions and microalgae diversity in the evaporation pond at the Saelices mining area versus a natural pond (Zamarra pond) in a near area but without mining activity.

| | Saelices evaporation | Zamarra pond |
|---|---|---|
| **Uranium concentration (mg $L^{-1}$)** | 25.0 | b.d.1 |
| **Pb (μg $L^{-1}$)** | 5.6 | <0.1 |
| **pH** | 3.4 | 6.9 |
| **Conductivity (mS cm-1)** | 10.8 | 0.2 |
| **Radioactivity (μSv $h^{-1}$)** | 4 | <0.1 |
| **Number of microalga species** | 4 | >25 |

(b.d.l. = below detection limit).

## 3. MICROALGAE ARE ABLE TO SURVIVE IN AN ENVIRONMENT CONTAMINATED BY URANIUM

Microalgae are very successful organisms, dominating a large number of habitats including extreme environments. Its biological characteristics (i.e. haploids, short generation times, enormous population size) allow them to adapt very quickly under environmental stress.

Extreme environments characterized by extreme values of stress factors (i.e. toxics, pH…) often support communities of phytoplancton living at the extreme limits of their tolerance (9-11). U is very hazardous for microalgae as a heavy metal besides its radioactivity. The high U concentration in Saelices evaporation pond (25 mg $L^{-1}$, around 700 times higher that $LC_{50}$ for microalgae species according US EPA Ecotox Database) indicates that these waters constitute extreme environments. Even in areas influenced by mining activities, the usual U concentration is less of 3.5 mg $L^{-1}$ (12).

It has generally been accepted that adaptation to extreme environments is achieved gradually by selection of several mutations with minor effects following a slowly process involving long temporal scales according to Charles Darwin's axiom '*natura non facit saltum*' (reviewed by Gould (13)). The early work of Goldschmidt suggested that unique mutations with large effect could have evolutionary importance, but evolutionary biologists overwhelmingly rejected Goldschmidt's work arguing that the multiple mutations of small effect are the pacemaker of adaptation (reviewed in Gould (13-14)). According to this conception, the organisms that live in these extreme environments are extremophiles that after getting their adaptation over millions of years differ very much from their mesophilic ancestors. However, adaptation of microalgae to extreme environment of uranium mining ponds Saelices could change this preconception.

Amazingly, some microalgae species were able to colonize this extremely U-contaminated pond very rapidly since mining activities started in 1960. There are few known cases where microalgae are able to colonize as quickly an extremely polluted environment, which make available a fascinating laboratory to study rapid adaptation of current microorganisms to extreme contamination.

Two Chlorophyta species (*Chlamydomonas fonticola*, *Dictyosphaerium ehrenbergianum*) and one Bacillariophyta species (*Pleurosigma acuminatum*) were detected in Saelices evaporation pond. Apparently microalgae from these ponds are common species that do not differ than those found in non-extreme locations.

To check this DNA fragments of *Chlamydomonas* were sequenced and compared with the N.C.B.I. database revealing as the closest specie discovered *Chl. fonticola* a current microalgae species, which live in unpolluted environments.

As expected numerous microalgae species proliferates during the yearly cycle in the Zamarra natural pond without mining activity, which serves as an indicator of natural pollution levels in that area before mining activity.

Consequently, the key question is: how could these microalgae adapt so quickly to an environment as extreme?

The first response with the microalgae are facing to environmental stress is a physiological response (i.e. acclimatization) due to modification of gene expression (15). Afterwards, when values of environmental stress exceed physiological capabilities, only a genetic response (i.e. mutations that confer resistance) can allow adaptation (16-19). The simplest genetic response is achieved by a single mutation in a single gene capable of conferring resistance. These simple mutations that confer resistance to a contaminant may occur spontaneously without the selective agent (e.g. uranium) facilitate their appearance (i.e. pre-selective pattern of appearance of mutations). By contrast, the selective agent may be facilitating mechanisms for the occurrence of these mutations that confer resistance (i.e. post-selective model).

The discussion on how the mutations occur (i.e. post-selective model where mutations occurs in response to selective agent producing a direct and specific adaptation *versus* pre-selective model where mutation occurs spontaneously by chance prior to selective agent exposure) was one of the great controversies of biology. Two prominent nuclear physicists Leo Szilard and Max Delbruck were the key to solve this biological problem employing a complex mixture of laboratory techniques and statistical analysis (20-21). But their complex procedures were not always understood and among the microbiologists even today the controversy remains.

To determine the mechanism by which mesophilic microalgae might be able to adapt to the extreme environment of uranium mine, we chose two strains of a microalgae species (*Chlamydomonas reinhardtii* Dangeard, strains ChlaA from algae culture collection of UCM) taxonomically similar than those living in Saelices, but isolated from pristine place that had never been subjected to uranium contamination. We employ a fluctuation analysis to determine the mechanisms (i.e. physiological acclimatization, pre-selective mutations, post-selective mutations…)

that allow rapid adaptation of mesophyle microalgae to extreme environment of the uranium pond (Figure 2).

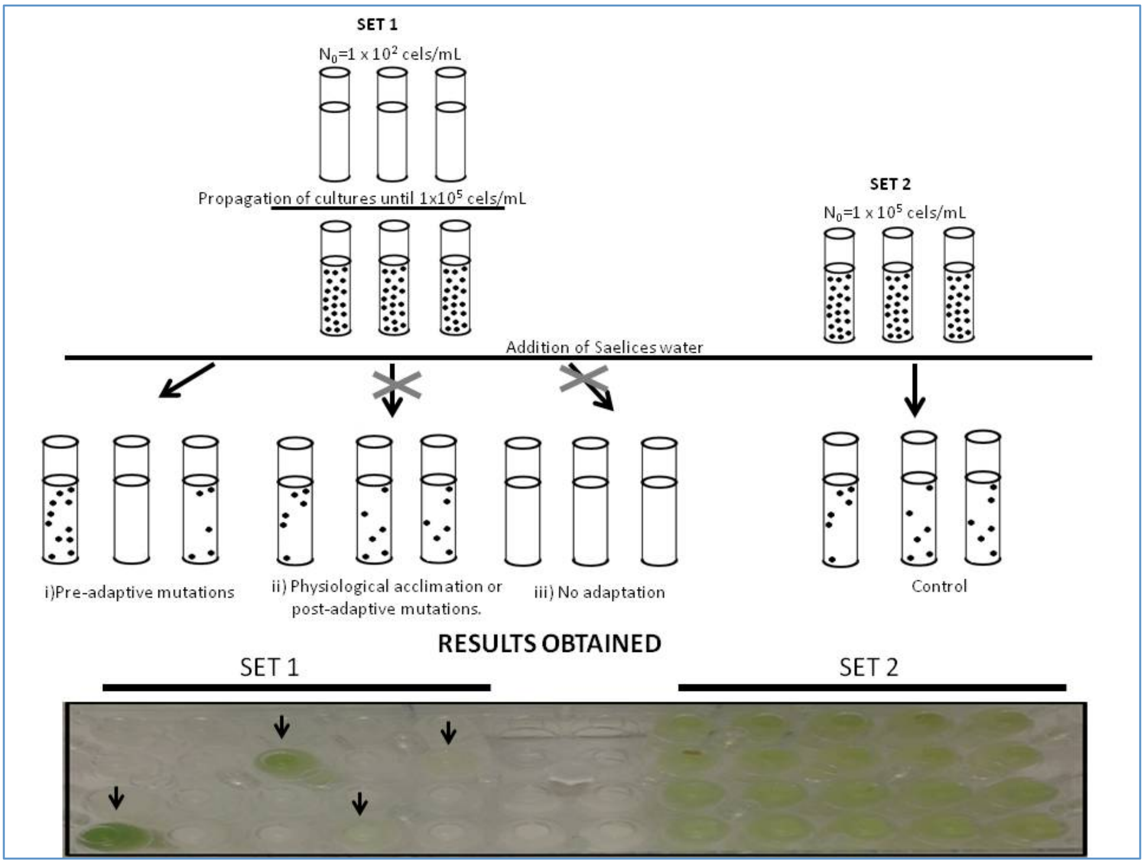


**Figure 2.- Schematic diagram of the possible results of a modified fluctuation analysis by Lopez-Rodas et al., (2001), from a classic Luria and Delbrück (1943) method.** Two sets of experiments were performed: in set 1, 96 cultures, founded from an initial inoculums of $N_0 = 10^2$ cells, were propagated until $N_t = 10^5$ cells without selective conditions and then, Saelices water enriched with $BG_{11}$ medium was added. The result obtained from set1 was i) adaptation thanks for rare pre-selective mutations occurred randomly before the exposure to Saelices water during the propagations of the cultures. The differences between the culture flasks depend in the early or late occurrence of the mutational event (arrows show the culture flasks that presented growth). The other two possible results of set1 are: ii) physiological acclimation or post-adaptive mutations (the cell number among the culture flasks must be similar) and iii) no adaption at all (empty culture flasks). In set 2, used as a control in other to sample the variance of the paternal population, the 30 replicates had initially $N_0 = 10^5$ cell from the same parental culture. The result obtained in set 2 was a similar number of resistance cells in all the cultures.

A massive destruction of cells was observed in each experimental culture of both sets 1 and 2 in the fluctuation analysis with Saelices evaporation pond water. However, after further incubation during 10 weeks proliferation of living resistant cells was observed in 7 cultures of set 1 experiments of *C. reinhardtii*. Simultaneously proliferation of resistant cells was also detected in all the cultures of set 2. The fluctuation in the number of resistant cells observed in set 1 experiment significantly exceeded the fluctuation observed in set 2 controls (set 1 cultures fluctuates from more than $10^5$ resistant cells $ml^{-1}$ to 0 resistant cells $ml^{-1}$, whereas all the set 2 cultures showed around $10^2$ resistant cells $ml^{-1}$).

Consequently, the U-resistant cells arose randomly by rare, pre-selective, spontaneous mutations and not as a response to the effect of residual waters from U mining (i.e. by specific physiological acclimatization, adaptive mutants or post-selective mutations). These U-resistant mutants were isolated and maintained in BG-11 medium in the absence of the selective agent (i.e. residual waters from U mining), then confirming that the culture was able to retain resistance to residual waters of U-mining throughout successive generations.

The rapid colonization by microalgae Saelices evaporation pond can be explained following an Occam's razor line of reasoning: i) new U-resistant mutants arise prior to the uranium exposure, because mutation is recurrent; ii) prior to the uranium exposure many of these U-resistant mutants are eliminated by selection or by chance; iii) The balance between new U-resistant genotypes appeared by mutation and the U-resistant genotypes eliminated by selection will determine that a small number of U-resistant mutants are presents within microalgae populations at a given time. When uranium contamination occurs, U-sensitive wild type cells die and only the U-resistant cells are able to colonize the new extreme environment (Figure 3).

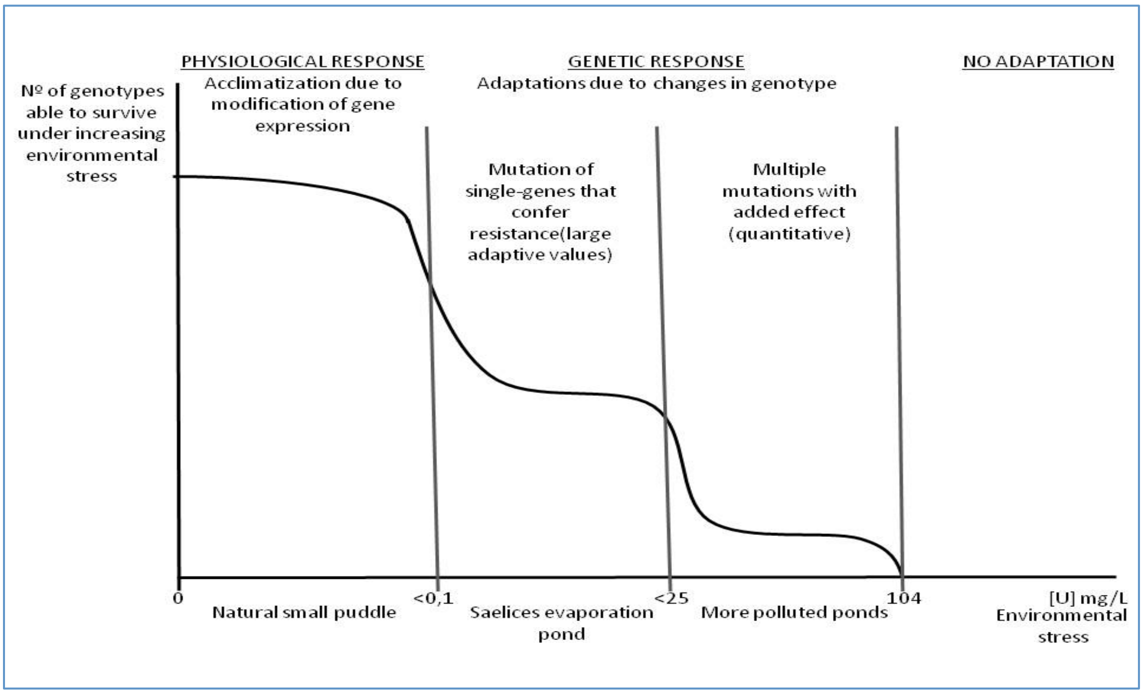


**Figure 3.-**Graphic work representation of the number of genotypes able to survive under an increasing environmental stress present among the studied ponds due to the uranium concentration and the adaption strategy depending in the uranium concentration.

Recent evidences suggest that mutations of single-genes that confer a large adaptive value do happen and, when competing with multiple small-effect mutations, they usually win (reviewed by Chouard (22)). In our experience over the past 10 years we have shown that a single mutation of large effect, which occur previously to selective agent exposure and are maintained in microalgae populations as mutation-selection balance allow adaptation of microalgae to pesticides (18, 23-25), xenobiotics (26), algaecides (27-28), mine spills (29-31) volcanic effluents and thermal waters (32-33).

## 4. MICROALGAE ARE ABLE TO CONCENTRATE URANIUM

Once it is found that microalgae can survive in an environment with large amounts of Uranium the next step for become able to build a natural nuclear reactor is that microalgae can to concentrate uranium. However, it not seems easy that microalgae could concentrate uranium because U has not knowledge biological utility. Consequently, we look for the possibility of uranium intracellular and extracellular sequestration in the *Chlamydomonas* cells collected from the Saelices evaporation pond (containing 25 mg $l^{-1}$ of uranium).

An Inductively Coupled Plasma-Mass Spectrometry (ICP-MS VARIAN RedTop) analysis demonstrated that *Chlamydomonas* cells bind a considerable total uranium quantity as 115 mg U $g^{-1}$ of dried biomass. Additionally, ICP-MS and EDX microanalysis confirmed the participation of two different mechanisms in the uranium removal: bio-adsorption to the cell wall (and bioaccumulation within the cell (Figure 4).

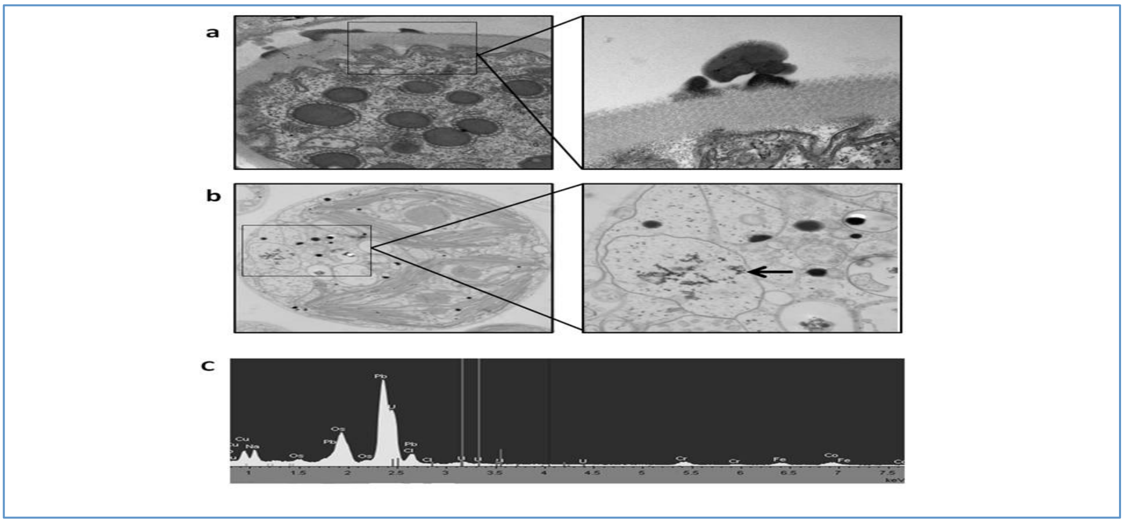


**Figure 4.-** TEM imagines, a) bio-adsorption in the cells wall, b) bio-accumulation of U inside the cells and c) TEM-XEDS analysis, in *Chlamydomonas sp.* Arrow detail in inlet showing uranium precipitates.

## 5. MICROALGAE ARE ABLE TO ENRICH URANIUM (PRODUCING ISOTOPIC FRACTIONATION $^{235}U/^{238}U$).

In all probability the hardest challenge to meet is that microalgae could get isotopic fractionation of uranium. However, after decades of study of stable isotopes in ecosystems is well known that biological enzymes sometimes produce isotopic fractionation (34).

As is well known, uranium found in nature consists largely of two isotopes, $^{238}U$ and the less abundant $^{235}U$, which constitutes only approximately 0.7% of naturally occurring uranium. Most of the commercial nuclear power reactors require uranium enriched in the $^{235}U$ (35).

Biological fractionation of U has been recently tried by an indirect procedure linking bacterial uranium reduction to the isotopic partitioning (36). However, one step uranium enrichment by preferential uptake of an isotope directly using a microorganism not been implemented yet. *Chlamydomonas* isolated from the uranium polluted site of Saelices, was tested for U fractionation. For this, the uranium isotopic composition in water of Saelices evaporation pond was compared with the isotopic ratio of U bio-accumulated by *Chlamydomonas* (previously the cells were treated with EDTA to eliminate metals on the cell wall). The analysis through a High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICPMS) let to obtain the isotopic distribution of uranium.

The results obtained reveal that *Clamydomonas* is able to U fractionation ($\delta^{235}U$ inside the cells with respect to $^{235}U$ in Saelices evaporation pond was -3.5 %). This spectacular result indicates that microalgae are capable of producing uranium fractionation effectively. A microalgae plant for uranium enrichment could be a real possibility.

## 6. CONCLUSIONS

Once more nature has won the race to the human technology: Microorganisms were able to adapt to extreme U contamination as well as accumulate and perform isotopic fractionation of uranium to become the most feasible creators of the first nuclear reactor in Oklo 1.7 billion years ago. The extreme environment of the evaporation pond in Saelices uranium mine provides some ideas of how microorganisms can build a nuclear reactor. Microalgae seem good candidates for nuclear engineers:

First of all they were able to adapt to the extreme U-contaminated environment of Saelices mining area in less than 40 years. These algae had suffered a pressure of selection in which pre-adaptive mutations that confer resistance against the toxic effect of uranium can survive.

Second efficient biological accumulation of uranium (115 mg U/g dry mass) was demonstrated.

Finally, microalgae are able to successfully carry out an isotopic fractioning of uranium.

## 7. ACKNOWLEDGEMENTS

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Part of this work is reflected in the Patent P201201203. The microalgae strain is deposited in the Deposit of Microorganisms for the Purposes of Patent Procedures no. BEAD04-12.

## 8. REFERENCES

1. Cowan, G. A. Natural Fission Reactor. Scientific American 235, 36–47 (1976).
2. Meshik, A. P. The Workings of an Ancient Nuclear Reactor. Scientific American. In press (2005).
3. Kuroda, P. K. On the Nuclear Physical Stability of the Uranium Minerals. Journal of Chemical Physics 25 (4), 781–782; 1295–1296 (1956).
4. Gauthier-Lafaye, F.; Holliger, P.; Blanc, P.L. Natural fission reactors in the Franceville Basin, Gabon: a review of the conditions and results of a "critical event" in a geologic system. Geochimica et Cosmochimica Acta 60 (25), 4831–4852 (1996).
5. Meshik, A. P.; Hohenberg, C. M.; Pravdivtseva, O.V. Record of Cycling Operation of the Natural Nuclear Reactor in the Oklo/Okelobondo Area in Gabon. Physical Review Letters 93 (18), 182302 (2004).
6. Lovelock, J.: *The ages of gaia: A biography of our living planet;* W.W. Norton; New York; 1998, 116-117.
7. Markich, S.J. Uranium speciation and bioavailability in aquatic systems: an overview. Sci. World J. 2, 707–729 (2002).
8. Arribas, A. Sobre el origen de las mineralizaciones españolas de uranio en rocas meta-sedimentarias. Bol. Geol. Min. 48, 705-711 (1987).
9. Costas, E.; Flores-Moya, A.; Perdigones, N.; Maneiro, E.; Blanco, J.L.; García, M.E.; López-Rodas, V. How eukaryotic algae can adapt to the Spain's Rio Tinto: A neo-Darwinian proposal for rapid adaptation to an extremely hostile ecosystem. New Phytol. 175, 334-339 (2007).
10. Costas, E.; Flores-Moya, A.; López-Rodas, V. Rapid adaptation of algae to extreme environments (geothermal waters) by single mutation allows "Noah's Arks" for photosynthesizers during the Neoproterozoic "Snowball Earth"?. New Phytol. 189, 922-932 (2008).
11. Seckbach, J.; Oren, A. *Oxygenic photosynthetic microorganisms in extreme environments*; In: Seckbach J. (ed.); Algae and Cyanobacteria in Extreme Environments; Springer; Dordrecht; 2007, 4-25.
12. Desssouki, Tarik C.E.; Hudson, Jeff J.; Neal, Brian R.; Bogard, Matthew J. The effects of phosphorus additions on the sedimentation of contaminants in a uranium mine pit-lake. Water Research 39, 3055-3061(2005).

13. Gould, S. J.; *The structure of evolutionary theory*; Belknap Press; Pg 1.433. (2002) ISBN: 0-674-00613-5.
14. Gould, S. J. The Return of Hopeful Monsters. Natural History 86: 24, 30 (1977).
15. Fogg, G. E. Algal adaptation to stress- some general remarks; In: *Algal adaptation to environmental stresses. Physiological, biochemical and molecular mechanisms;* Rai, L.C.; Gaur, J.P.; eds.; Springer; Berlin, Germany: 2001; 1–20.
16. Sniegowski, P. D.; Lenski, R.E. Mutation and Adaptation: the directed mutation controversy in evolutionary perspective. Annual review of ecology and systematic 26, 553-578 (1995).
17. López-Rodas, V.; Agrelo, M; Carrillo, E.; Ferrero, L. M.; Larrauri, A.; Martínotero L.; Costas, E. Resistance of microalgae to modern water contaminants as the result of rare spontaneous mutations. European Journal of Phycology 36, 179-190 (2001).
18. Costas, E.; Carrillo, E.; Ferrero L. M.; Agrelo, M.; García-Villada, L.; Juste J.; López-Rodas, V. Mutation of algae from sensitivity to resistance against environmental selective agents: The ecological genetics of *Dictyosphaerium chlorelloides* (Chlorophyceae) under lethal doses of 3-(3,4-dichlorophenyl)-1,1- dimethylurea herbicide. Phycologia 40, 391-398 (2001).
19. Sniegowski, P.D. Linking mutation to adaptation: overcoming stress at the spa. New Phytologist 166, 360-362 (2005).
20. Luria, S.E.; Delbrück M. Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28, 491-511 (1943).
21. Novick A.; Szilard L. Description of a Chemostat. Science 112, 2920:715-716 (1950). DOI: 10.1126/science.112.2920.715
22. Chouard, T. Revenge of the hopefull monster. Nature 463, 7283:864-867 (2010).
23. López-Rodas, V.; Flores-Moya, A.; Maneiro, E.; Perdigones, N.; Marva, F.; García, M.E.; Costas, E. Resistance to glyphosate in the cyanobacterium *Microcystis aeruginosa* as result of pre-selective mutations. Evol. Ecol. 21, 535-547 (2007).
24. Marva, F.; Lopez-Rodas, V.; Rouco, M.; Navarro, M.;Toro, F. J.; Costas, E.; Flores-Moya, A. Adaptation of green microalgae to the herbicides simazine and diquat as result of pre-selective mutations. Aquatic Toxicology 96,130–4 (2010).
25. Gonzalez, R.; García-Balboa, C.; Rouco, M.; Lopez-Rodas, V.; Costas, E. Adaptation of microalgae to lindane: A jew approach for bioremadiation. Aquatic Toxicology 109, 25-32 (2012).
26. García-Villada, L.; López-Rodas, V.; Bañares-España, E.; Flores-Moya, A.; Agrelo, M.; Martín-Otero, L.; Costas, E. Evolution of microalgae in highly stressing environments: An experimental model analyzing the rapid adaptation of *Dictyosphaerium chlorelloides* (Chlorophyceae) from sensitivity to resistance against 2,4,6-trinitrotoluene by rare preselective mutations. Journal of Phycology 38,1074-1081(2002).
27. García-Villada, L.; Rico, M.; Altamirano, M.; Sánchez-Martín, L.; López-Rodas V.; Costas, E. Occurrence of copper resistant mutants in the toxic cyanobacteria *Microcystis aeruginosa*: Characterization and future implications in the use of copper sulphate as an algaecide. Water Research 38, 2207-2213(2004).
28. Costas, E.; Gonzalez, R.; Lopez-Rodas, V.; Huertas E. Mutation of microalgae from antifouling sensitivity to antifouling resistance allowa phytoplankton dispersal through ships´ biofouling. Biol. Invasions (2013). DOI 10.1007/s10530-012-0405-8.
29. Baos, R.; Garcia-Villada, L.; Agrelo, M.; López Rodas, V.; Hiraldo, F.; Costas, E. Short-Term adaptation of microalgae in highly stressfull environments: an experimental model analysing the resistance of *Scenedesmus intermedius* (Chlorophyceae) to the heavy metals mixture from the Aznalcollar mine spill. Eur. J. Phycol. 37, 593-600 (2002).
30. Lopez-Rodas, V.; Marvá, F.; Costas, E.; Flores-Moya, A. Microalgal adaptation in the stressful acidic, metal-rich mine waters from Mynydd Parys (N Wales, UK) could be due to

selection of pre-selective mutants. Environmental Experimental Botany 61, 43-48 (2008a).

31. Lopez-Rodas, V.; Marvá, F.; Rouco, M.; Costas, E.; Flores-Moya, A. Adaptation of the chlorophycean *Dictyosphaerium chlorelloides* to the stressful acidic, mine metal-rich waters from Aguas Agrias Stream (SW Spain) as result of pre-selective mutations. Chemosphere 72, 703-707 (2008).
32. Lopez-Rodas,V.; Perdigones,N.; Marvá,F.; Maneiro,E.; Rouco, M.; Delgado, A.; Flores-Moya, A. & Costas, E. Living in Vulcan´s forge: Algal adaptation to stressfull geothermal ponds on Vulcano Island (southern Italy) as result of pre-selective mutation. Phycological Research 57:111-117 (2009).
33. Costas, E.; Flores-Moya, A.; López-Rodas, V. Rapid adaptation of phytoplankters to geothermal waters is achieved by single mutations: were extreme environments 'Noah's Arks' for photsynthesizers during the Neoproterozoic 'snowball Earth'? New Phytol. 180, 922–32 (2008).
34. Hayes, I. M. (1982). Fractionation: an introduction to isotope measurements and terminology. Spectra 8, 3–8
35. Czerwinski, K. R. & Martin F. P. Uranium enrichment using microorganisms. Patent nº: US 7,452,703 B1. United States (2008).
36. Basu, A.; Sanford, R.; Johnson, T.M. & Lundstrom, C. Microbial Uranium reduction: Isotopic fractionation factors for bacteria with diverse metabolism. PhD Tesis, University of Illinois at Urbana-Champaign, 83-113 (2012).