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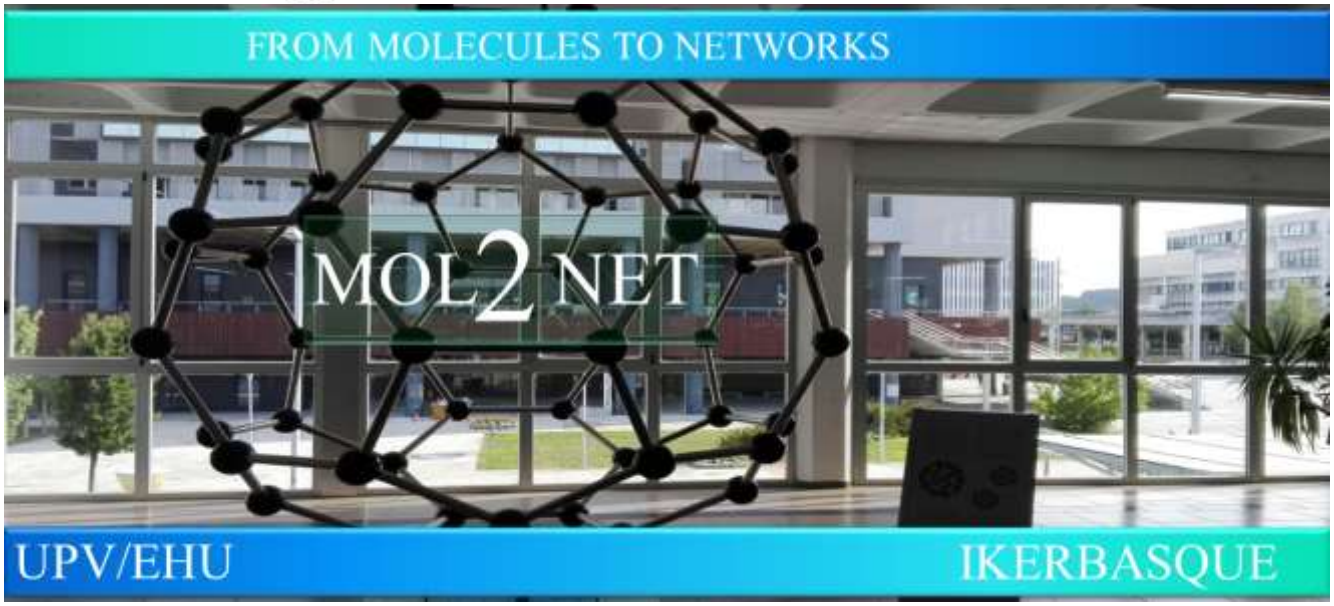

Effect of the Cd/Ca interaction on the growth of *Paramecium* sp acclimated on two (optimal and low) temperatures

FERFAR, M.[a], *BOUGHOULA, R.*[b], *DRIOUCHE, Y.* [a] , *BOUHROUM, F.*[b] *and DJEBAR, M.R.*

[a] *Environmental Research Center, Sidi Amar Campus, BO: 2024, Annaba, 23005, Algeria.*
[b] *Cellular Toxicology Laboratory, Department of Biology, Faculty of Science, Badji Mokhtar University Annaba, BP 12, 23000, Annaba, Algeria.*
m.ferfar@cre.dz

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Graphical Abstract	**Abstract.**
Insert grafical abstract figure here	*Temperature changes affect all physiological processes, such as modification of membrane fluidity and local transitions of the lipid bilayer, which can affect membrane integrity and permeability, as well as the mobility and function of membrane receptors.* *This research studies the effect of temperature*

	variations and Cd/Ca interaction, on the growth of Paramecium sp. The concentrations of Cadmium Chloride were used (0.5, 1, and 10 µM) in addition to two concentrations of Calcium Chloride (20 µM and 2 mM). Paramecia were acclimated at 28° (the optimal temperature) and at 16° (low temperature). *The results show that temperature variations affect the growth of paramecia by slowing down their multiplication and affecting the absorption and bioaccumulation of the toxic metals present.* *At the same time, the presence of calcium in high concentrations strongly reduces the observed effects of cadmium.*

Introduction *(optional)*

Exposure to harmful environmental factors such as industrial discharges (e.g. heavy metals) and climate change determine species abundance, distribution and diversity. To cope with these stressors, organisms develop molecular mechanisms to adapt and survive.

The objective of this research is to study the effect of low concentrations of Cadmium in the presence or absence of low and high concentrations of calcium on the growth of an aquatic organism; Paramecium sp, acclimatized to variable temperatures: 16° and 28 °C.

Materials and Methods *(optional)*

The biological material used in our work is a freshwater unicellular microorganism, represented by: *Paramecium sp.*

For the heavy metal, we used Cadmium chloride hemipentahydrate. Its chemical formula is: CdCl25H2O, and for calcium we used Calcium Chloride Dihydrate. Its chemical formula is: CaCl22H2O.

To better study the Cd/Ca interaction we chose several concentrations of Cadmium Chloride (0.5µM, 1µM, 10µM), and two concentrations of Calcium Chloride: a Low concentration CaL (20µM), and a High concentration CaH (2mM).

We proceeded to 3 tests at 16° and 28° C:

- The first in the presence of Cadmium, and absence of Calcium.
- The second, the control and the different concentrations of Cadmium in the presence or not of the low concentration of Calcium.
- The third, the control and the various concentrations of Cadmium in the presence or not of the strong concentration of Calcium.

Than we proceed to measure the Growth kinetics: by daily cell count according to the method of Sauvant et al, (1999). The observation is done by microscope (Leïca DL 1000).

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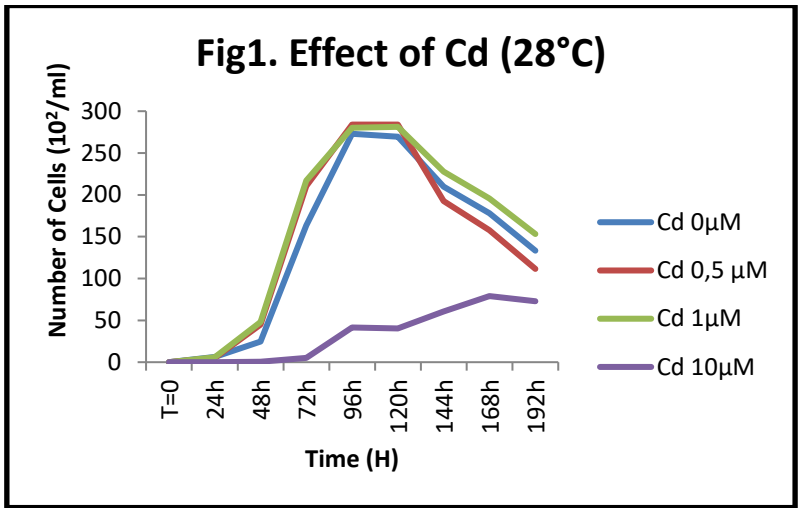
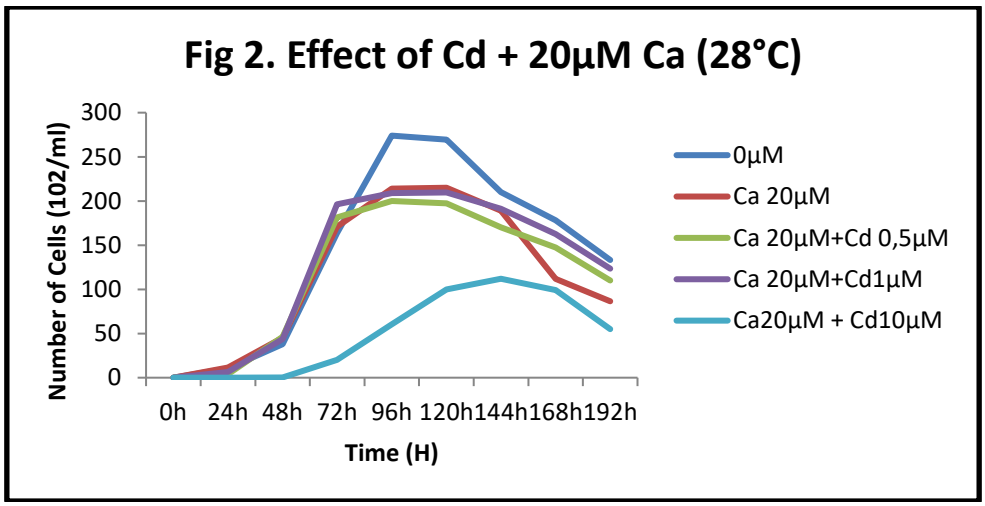
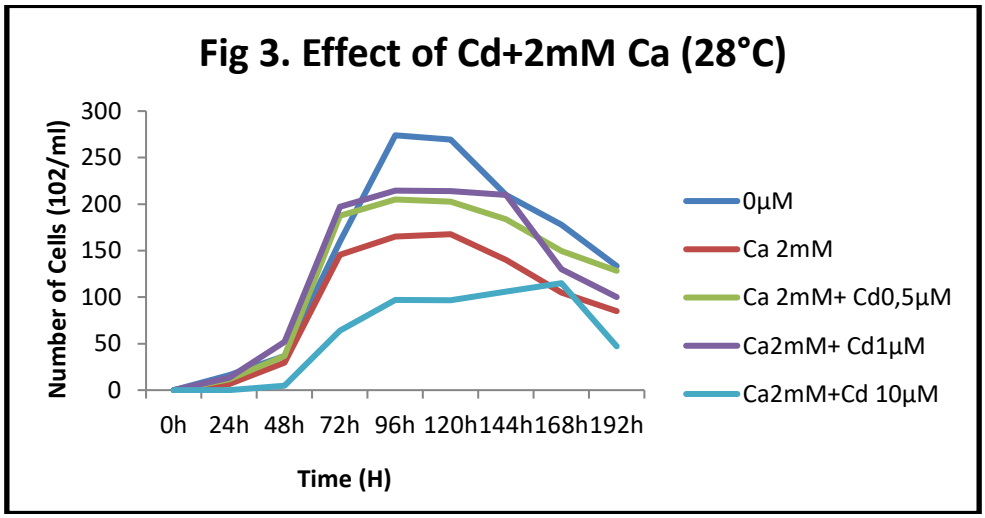
Results and Discussion *(optional)*







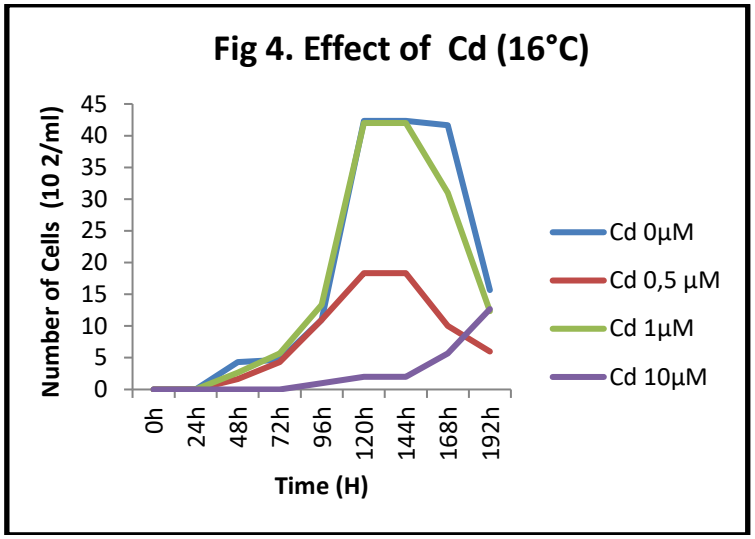


Fig 4. Effect of Cd (16°C)
Number of Cells (10 2/ml)
45
40
35
30
25
20
15
10
5
0
0h
24h
48h
72h
96h
120h
144h
168h
192h
Time (H)
Cd 0µM
Cd 0,5 µM
Cd 1µM
Cd 10µM

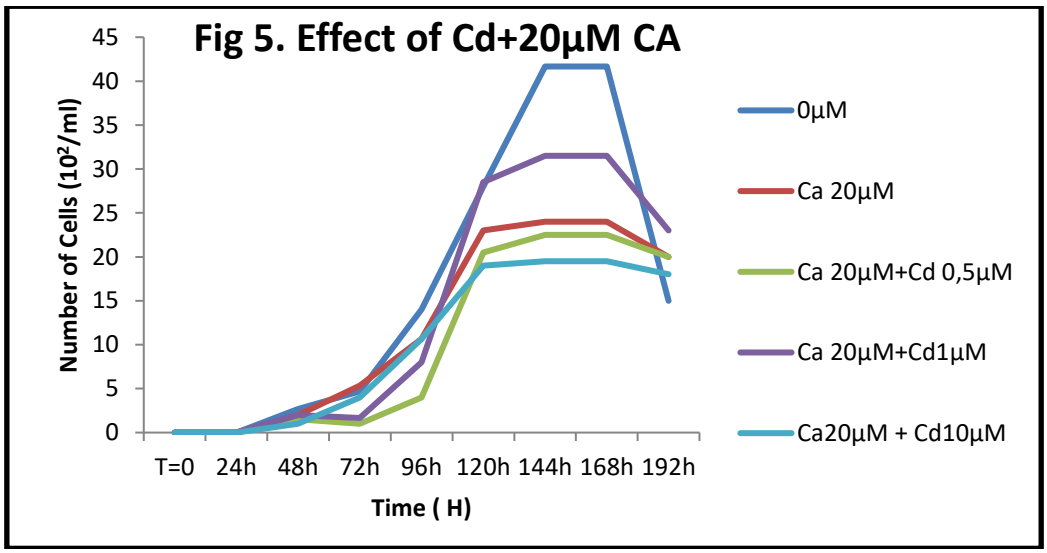


Fig 5. Effect of Cd+20µM CA
Number of Cells (10²/ml)
45
40
35
30
25
20
15
10
5
0
T=0
24h
48h
72h
96h
120h
144h
168h
192h
Time (H)
0µM
Ca 20µM
Ca 20µM+Cd 0,5µM
Ca 20µM+Cd1µM
Ca20µM + Cd10µM

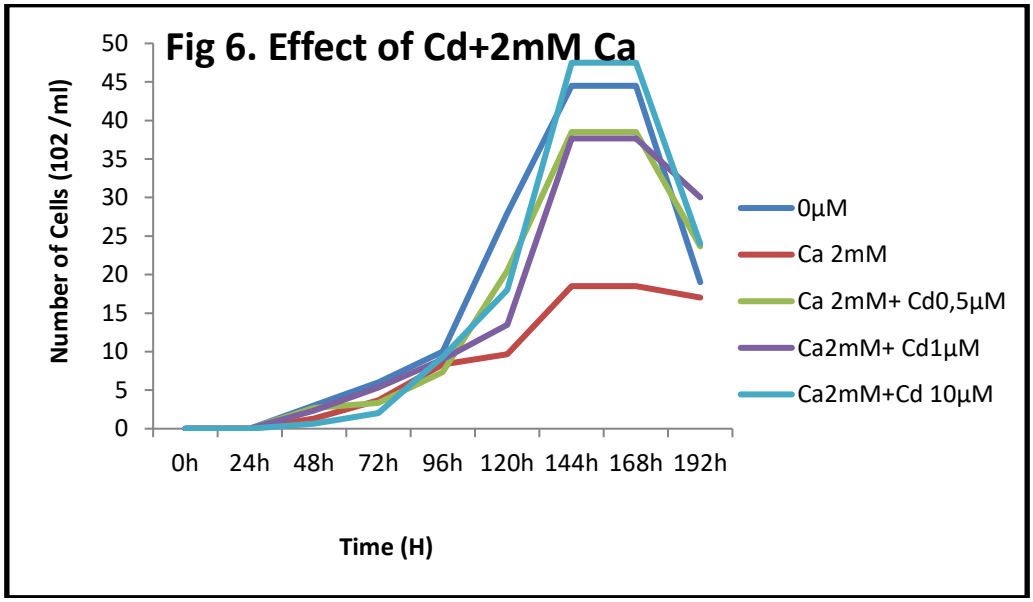


Fig 6. Effect of Cd+2mM Ca
Number of Cells (102 /ml)
50
45
40
35
30
25
20
15
10
5
0
0h
24h
48h
72h
96h
120h
144h
168h
192h
Time (H)
0µM
Ca 2mM
Ca 2mM+ Cd0,5µM
Ca2mM+ Cd1µM
Ca2mM+Cd 10µM

Growth curves provide quantitative data for a reliable analysis of the toxic effect of a given substance.

1.Effect of cadmium on cell growth

Figures 1 and 4, illustrate the time-dependent effect of increasing cadmium concentrations on the growth kinetics of *Paramecium sp* at 28°C and 16°C

The results obtained for the culture at 28°C show a latent phase in the control and treated paramecia between 0 and 24h in which growth is null. From 24h to 48h, we observe an increase in the number of control and treated cells with increasing concentrations of cadmium (0.5µm; 1µM), while for the 10µM concentration of cadmium the growth is inhibited.

After 48 h, we observe a growth superior to that of the controls in the cells treated with the concentrations of cadmium (0.5µM and 1µM). At the same time, cell growth is strongly inhibited by 10µM of cadmium compared to controls. This increase continues as a function of time until 96 h.

From 96 h to 120 h. The number of cells treated with increasing concentrations of cadmium (0.5µM; 1µM; 10µM) show a steady state.

From 120 h, the number of control cells and cells treated with cadmium concentrations (0.5µM; 1µM) progressively decreases. At the same time, we observe an increase in the number of cells treated with the 10µM concentration of cadmium, beyond 168 h the number starts to decrease.

While for the culture at 16°C, we observe a latent phase in the control and treated paramecia until 48 h.

After 48 h, the cell growth increases in the control paramecia. We also note that for the concentration of 1µM of cadmium the evolution of cell growth seems similar to that of the control cells. Thus, for cadmium concentrations (0.5µM and 10µM) we observe a weak growth compared to the controls. This increase continues as a function of time until 120 h (exponential phase). From 120 h to 144 h, the number of cells treated with increasing concentrations of cadmium (0.5µM; 1µM; 10µM) show a stationary state.

From 144 h, the number of control and treated paramecia with the concentrations (0.5µM and 1µM) progressively decreases, in parallel the number of cells treated with the continuous 10µM cadmium concentration increased.

2.Effect of cadmium on cell growth in the presence of 20µM Calcium

Figures 2 and 5, represent the effect of the combined treatment of cadmium and calcium on the evolution of the growth of Paramecia at 28°C and 16°C as a function of time.

The results obtained for the culture at 28°C, show a latent phase in the control and treated paramecia between 0 and 24 hours. From 24 h, we observe a normal growth with an exponential phase in the control paramecia which reaches its maximum at 96 h. At the same time, we observe a growth inhibition in cells treated with 20µM of calcium only and in those treated with 0.5µM and 1µM of cadmium in the presence of 20µM of calcium compared to the controls, thus for the cells treated with 10µM of cadmium in the presence of 20µM of calcium, the growth is strongly inhibited compared to the controls.

From 96 h to 120 h, control and 20µM calcium only treated cells and 0.5µM and 1µM cadmium concentrations in the presence of 20µM calcium show a stationary growth state, which is followed by a decrease in cell number. While the number of cells treated with 10µM cadmium in the presence of 20µM calcium, increases continuously until 144 h, then decreases progressively.

While, for the culture at 16°C, the cells show a latent phase from 0 to 48 h in which the growth is null. From 48 h, a normal growth with an exponential phase in the control paramecia. At the same time, we note a weak growth compared to the controls for the cells treated with increasing concentrations of cadmium (0.5µM; 1µM and 10µM) in the presence of 20µM of calcium and the cells treated with 20µM of calcium only. This increase continues as a function of time until 144 h. From 144 h to 168 h, both control and treated cells show a stationary growth state, which is followed by a decrease in cell number.

3. Effect of cadmium on cell growth in the presence of 2mM Calcium

Figures 3 and 6, represent the effect of increasing concentrations of cadmium in the presence of 2mM calcium on the growth evolution of paramecia at 28°C and 16°C, as a function of time.

The results obtained for the culture at 28°C, show a latent phase in the control and treated paramecia between 0 and 24 hours. From 24 hours, we observe a normal growth with an exponential phase in the control paramecia which reaches its maximum at 96 hours, we record a number of 27400 paramecia / ml. At the same time, the evolution of cell growth in paramecia treated with 2mM calcium only and the concentrations 0.5µM and 1 µM cadmium in the presence of 2mM calcium is inhibited compared to the controls, so for the cells treated with the concentration 10 µM cadmium in the presence of 2 mM calcium, the growth is strongly inhibited compared to the controls

The control and treated cells show a stationary growth state, from 96 hours to 120 hours, which is followed by a progressive decrease in the number of cells treated with 2 mM calcium only and the concentrations 0.5 µM and 1 µM cadmium in the presence of 2 mM calcium. At the same time, we observe an increase in the number of cells treated with 10 µM cadmium in the presence of 2 mM calcium, from 168 hours the number starts to decrease.

While, for the culture at 16°C, the cells show a latent phase from 0 to 48 hours in which the growth is null. From 48 hours, we observe a normal growth with an exponential phase in the control paramecia which reaches its maximum at 144 hours, we record a number of 4400 paramecia / ml. In the cells treated with 10µM of cadmium in the presence of 2mM of calcium, the growth is higher than the control which reaches a number of 4700 paramecia / ml. As for the cells treated with the concentration 0.5 µM and 1 µM of cadmium in the presence of 2 mM of calcium, we note a weak growth compared to the controls. While for the paramecia treated with the concentration 2mM of calcium only we see a strong inhibition of growth compared to the controls.

From 144 h to 168 h, both control and treated cells show a stationary growth state, which will be followed by a progressive decrease in cell number.

According to our results, we observe a significant growth for paramecia acclimated at 28°C compared to those acclimated at 16°C. Thus the presence of 2mM Calcium alone, inhibits the growth compared to the presence of 20µM Calcium. While the paramecia treated with Cadmium at 28°C and 16°C in the presence of 2mM Calcium, the number of cells is higher than that treated in the presence of 20µM Calcium.

Discussion

We were first interested in the effects of Cadmium in the presence and absence of Calcium on the growth of Paramecium sp, which is a key parameter **(Perez-Rama, 2001).** Growth has been widely used as an indicator of stress in aquatic environments **(Akcha, 2000; Amara, 2012)** because it provides an estimate of the impact of a xenobiotic. The growth assay involves measuring the growth kinetics of a microorganism in an artificial culture medium during storage under controlled conditions for a predefined time **(Branger et al., 2007).** The cell density in the medium is measured daily by direct cell counting.

Our results show that the number of paramecia grown at 28°C is higher than that of paramecia grown at 16°C. This means that a significant lowering of the environmental temperature affects the growth, inducing a slowing down or a total stop of the growth of the microorganisms, during which most of the protein synthesis is blocked. This latent phase, described as the "cold acclimation phase", allows the microorganisms to set up the mechanisms necessary to adapt to cold **(Graumann and Marahiel, 1996; Hebraud and Potier, 1999).** In which the cell produces cold shock proteins, CSPs, also known as class I proteins, whose expression level increases significantly during a short period of time, allow the adaptation of ribosomes to the translation of mRNAs coding for housekeeping proteins (proteins essential for basic cellular functions). The synthesis of these CSPs decreases, and a new protein is expressed. These "cold acclimation proteins", CAPs or class II proteins are expressed in a more moderate way and their level does not drop like that of the CSPs. The cell is said to be acclimatized to the cold, the protein synthesis of the housekeeping genes and the growth resume with a slower rate **(Pandiani F, 2010).**

Thus, when the temperature is low the fluidity becomes less than the optimal range and membrane activities are limited. However, the functions performed by the plasma membrane require an optimum fluidity. To maintain optimal fluidity, adaptive changes in lipid composition are required, such as an increase in unsaturated fatty acids, a decrease in fatty acid chain length, an increase in aniso-branched fatty acids and a decrease in iso-branched fatty acids, which largely compensate for the direct effects of temperature on membrane fluidity. This adaptation is called the homeoviscous adaptation **(Jeffrey R, 1995)**. As well as an increase in the ratio of phosphatidyl ethanolamine / phosphatidyl choline (Homeophase adaptation) **(Hazel, 1995)**. These adaptations allow to keep the structure of the membrane at low temperature **(Hazel, 1995)**. According to most studies, these modifications help preserve the functions of proteins embedded in membranes following a drop in temperature **(Hochachka and Somero, 2002).**

In addition, temperature affects the tolerance of aquatic organisms to metals **(Mubiana and Blust, 2007).** Studies have shown that temperature variation is accompanied by an increase in the uptake and bioaccumulation of metals **(Mubiana and Blust, 2007; Yang et al., 2007)**, which can lead to the mortality of exposed organisms **(Heugens et al., 2003).**

On the other hand, we observed a stimulation of cell growth for the two lowest concentrations of Cadmium, as well as an inhibition for the high concentration in paramecia treated at 28° C and 16° C. These results are similar to

that of **Eaton and Klaassen, (2001).** This stimulation can be explained by the phenomenon of "Hormesis", which can be defined as a process in which exposure to a low dose of a chemical agent or environmental factor that damages at higher doses induces an adaptive beneficial effect on the cell or organism **(Calabrese, 2007; Mattson, 2008).** In addition, Hormesis appears to be executed by a variety of physiological cellular processes that converge on enhanced stress resistance and longevity. For example, mild heat stress in flies leads to increased expression of stress response proteins, including heat shock proteins that are detectable after more than a week after treatment **(Sarup, 2014)**.

Thus, results showing inhibition of paramecium growth in the presence of 2 mM compared to those in the presence of 20 µM. Studies have shown that a regulated increase in calcium is a key signal in all cell types **(Berridge M, 2002; Bootman M et al., 2002)**, but an unregulated elevation of Calcium is often cytotoxic **(Annunziato L et al., 2003)**

Several pathways have been proposed to be involved in the transport of Cadmium into cells. It can be taken up by facilitated diffusion or active transport.

Transporters and receptors for free and complexed forms of essential metals such as Ca^{2+}, Fe^{2+}, Zn^{2+} or Cu^{2+}, can mediate Cadmium uptake **(Bridges et al., 2005; Thévenod, 2010). Usai et al, (1999)** showed that the pathways of entry of Cadmium is similar to that of Calcium and Calcium influx was indistinguishable from Cadmium influx in the presence of extracellular Calcium, **(Marchi et al., 2000).**

Therefore, Cadmium-induced cytotoxicity was related to the ability of the metal to induce Calcium influx **(Kazantzis, 2004; Yang, 2008).**

Cadmium can eventually be taken up by voltage-dependent Ca^{2+} channels or receptors, and inhibition of Ca^{2+} channels can sometimes protect against Cadmium toxicity. The calcium channel blocker nimodipine produced a shift in the LD50 of Cadmium from 15 to 45 µM in the pituitary cell line. Thus calcium protected the cells against the cytotoxic effects of Cadmium (**Niyogi and Wood, 2004)**. Our results show an increase in the growth of cells treated with Cadmium in the presence of 2 mM Calcium compared to those treated with 20 µM Calcium.

Conclusions

This research work aims to evaluate the effects of temperature and trace metals (Cadmium) in the presence and absence of Calcium on the physiology of paramecia.

The results obtained show that a lowering of the temperature affects the growth by inducing a slowing down of the latter, which would allow the paramecia to produce cold shock proteins "CSPs and CAPs" to resume the synthesis of household proteins associated with a restart of the growth.

We also observed that low concentrations of Cadmium have a stimulating effect. It also seems that the presence of high concentrations of Calcium would protect the cells against the cytotoxic effects of Cadmium. Therefore, an understanding of the effects of multiple stressors, such as pollutants and temperature and their mechanisms on physiological, biochemical, metabolic, morphological and behavioral parameters is essential to predict tolerance limits, survival of paramecia and to model the effects of global climate change on aquatic ecosystems worldwide.

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