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Growth Characteristics, Metal Uptake and Expression Analysis of Copper Metallothionein in a Newly Reported Ciliate, *Tetrahymena farahensis*

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Key words
Copper metallothionein, Copper uptake, Transcripts level, *Tetrahymena farahensis*, Ciliate protozoan

ABSTRACT

The present study describes the role of a novel metallothionein in copper homeostasis in a newly discovered ciliate *Tetrahymena farahensis*. The ciliate showed optimum growth at 27±1°C and pH 7.0 to 7.5. The maximum resistance dose of copper for *T. farahensis* was found to be 8, 9 and 80 µg/ml in wheat grain medium, Bold-basal salt medium and modified Neff's medium, respectively. *T. farahensis* exhibited significant copper storage ability removing 54.9 % copper from medium within 96 h of 30 µg/ml copper stress. The maximum uptake rate was observed within 30 min of copper administration in response to 5-100 µg Cu^{++}/ml in the medium. Quantitative analysis showed that *TfCuMT* is a copper inducible gene, the basal transcriptional level of which increased within 15 min of 10 µg/ml copper exposure upto 87.3 folds that later on decreased. Accordingly, maximum uptake rate was observed between 15 to 30 min of copper exposure indicating possible role of *TfCuMT* as copper metallothionein. *T. farahensis* and *TfCuMT* can be used as biotechnological tools for bioremediation.

INTRODUCTION

Metals are the basic component of our daily balanced diet and biomass. Some metals have specific role in metabolism, while others have no biological role (Lovley 2000). Some heavy metals considered as the most persistent inorganic pollutants tend to accumulate in biomass due to their non-degradable nature (Kong *et al*., 1995). Copper has been found to be more toxic to life compared with many other metals such as mercury, cadmium and zinc (Madoni *et al*., 1994).

Like other organisms, ciliates have developed mechanisms to cope with higher concentration of metal ions and maintain their physiological activities (Boldrin *et al*., 2002). Ciliates can survive longer in highly polluted water, indicating their ability to detoxify or overcome metal toxicity (Shakoori *et al*., 2004; Rehman *et al*., 2007). In heavy metal contaminated water, ciliates though show reduced growth, exhibit ability to remove up to 98 % of the heavy metal ions from the wastewater (Rehman *et al*., 2008, 2009). These characteristics of ciliates (metal removal from medium and long term survival) open up new avenues for their use in bioremediation of industrial wastewater (Shakoori *et al*., 2004).

In eukaryotes, the heavy metal resistance mechanisms involve biosorption, bioaccumulation and biotransformation (Vieira and Volesky, 2000). Bioaccumulation is an important tool of resistance against heavy metals. It is the most studied mechanism among ciliates (Gutierrez *et al*., 2011). In animals and protists bioaccumulation of heavy metals is based on glutathione and metallothioneins (MTs) (Binz and Kägi, 1999). These proteins bind with metal ions and are accumulated in vacuoles and later released as metallic complex (Diaz *et al*., 2006). MTs have greatest binding capacity with Cu followed by Cd and Zn (Kägi and Kojima, 1987).

MTs are induced by a variety factors including metals (Cu, Cd, Hg and Zn), cytokines and reactive oxygen species

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(ROS) (Thirumoorthy *et al*., 2011). Zn and Cu are regarded as the primary physiological inducers. Recently some of us have described copper metallothionein gene from a newly discovered *Tetrahymena* species from Pakistan, *T. farahensis* (Zahid *et al*., 2016). In present study, we are describing the effect of different concentrations of copper ions on growth of *Tetrahymena farahensis* and transcription of a copper metallothionein gene *TfCuMT* (Acc. # HE820725) of the same strain and correlating the transcripts level with the copper uptake rate/storage ability. The longer survival of ciliates in metal contaminated environment as compared to bacteria make them a better choice for bioremediation. Study of metallothioneins involved may lead to better understanding of resistance mechanisms. Transformation of microbes with such genetic determinants may also help in increased metal uptake and environmental clean-up ability.

MATERIALS AND METHODS

Isolation and maintenance of ciliates

Tetrahymena farahensis isolated from the industrial wastewater in 2010 (Zahid *et al*., 2014) is still being maintained in Protozoan Culture Laboratory, School of Biological Sciences, University of the Punjab, Lahore in Bold-basal salt medium (Shakoori *et al*., 2004). The algal, bacterial or fungal contamination (if occurred) in the growing cultures was eliminated by keeping the cultures in dark and adding antibiotics including kanamycin (50 µg/ml)/ ampicillin (100 µg/ml)/ chloramphenicol (20 µg/ml) and fungicide fungizone/ amphotericin B (1 µg/ml) (Zahid *et al*., 2014). Copper resistant *T. farahensis* was isolated by adding $CuSO_4$ (stock Conc. 1 mg/ml) with final concentration of 1 µg Cu^{++} /ml in two days old culture in Bold-basal salt medium. The addition of copper at the same rate was continued for next two days. The surviving organisms were considered as metal resistant.

Determination of growth characteristics

Growth pattern of *T. farahensis* was determined in Bold-basal salt medium (Shakoori *et al*., 2004), wheat grain medium (Rehman *et al*., 2006) and modified Neff's medium (Cassidy-Hanley *et al*., 1997). For each growth curve, 50 cells of *T. farahensis* were inoculated in 30 ml of the medium in 100 ml conical flask and cultures were grown up to 8 days. Culture sample (10 µl) was taken out at regular intervals after every 24 h, and number of cells was counted under microscope at a magnification of 50 X using hemocytometer. All experiments described in this article were performed in triplicate.

Different temperatures and pH were used to determine optimum conditions for ciliate growth in each of the three media used. To determine the effect of temperature, cells were grown at 20, 27, 37 and 42°C in media with pH 7.2±0.1. Optimum pH was determined by growing *T. farahensis* culture at pH 6.0, 6.5, 7.0, 7.5 and 8.0 at 27±1 °C. Growth curves were prepared from cell count that was recorded regularly after every 24 h for 7-8 days.

Determination of effect of copper on growth

Growth curves were also prepared in the presence of Cu^{++}. $CuSO_4$ was added in one day old cultures of *T. farahensis* at the rate of 1 µg Cu^{++}/ml/day (15.75 µM Cu^{++}/ml/day) in Bold-basal salt and wheat grain media and 10 µg Cu^{++}/ml/day (157.5 µM Cu^{++}/ml/day) in modified Neff's medium and growth curves were prepared. Maximum resistance/tolerance dose, the highest metal concentration at which a few cells survived that can be used for restoration of population was also measured in each medium.

Effect of different concentrations of the metal, *viz.,* 1, 5 and 10 µg/ml/day of copper ions was determined on the growth of *T. farahensis* in modified Neff's medium. One day old ciliate cultures were used for this purpose. Growth curves were prepared to evaluate the effect of increasing concentration of copper on growth of the cells.

Determination of Cu uptake ability

Copper uptake from the medium containing only one concentration of Cu^{++}:

Three sets, each of three 250 ml conical flasks with 100 ml modified Neff's medium were used for determination of copper uptake ability of *T. farahensis*. Each set consisted of three flasks *viz*., flask 1(experimental), flask 2 (negative control) and flask 3 (positive control). Flask 1 (experimental) and 2 (negative control) were inoculated with 50 cells of well grown culture of *T. farahensis.* Next day, copper ions at 30 µg/ml were added to flask 1 (experimental) and flask 3 (positive control). An aliquot (1 ml) was taken from each flask at 0, 24, 48, 72 and 96 h, and centrifuged at 2340 xg for 10 min. Supernatants were saved at -20 °C and the pellets were washed with 1 ml of saline solution (0.9 % NaCl). Air dried pellets were dissolved in 50 µl of Conc. HNO_3 and final volume of digested pellets was made up to 1 ml with deionized water. Air acetylene flame in Thermo Unicam-Solaar atomic absorption spectrophotometer was used to find the metal concentration in each pellet and supernatant indicating copper uptake by *T. farahensis* and copper ions remaining in the medium, respectively.

Copper uptake from the medium containing different concentrations of Cu^{++}:

In another set of experiment, 3 days old culture of *T.*

farahensis in 30 ml of modified Neff's medium was used for determination of uptake ability of the ciliate under different concentrations of copper stress *viz*. 5, 10, 50 and 100 µg/ml. The positive (with metal but no ciliates) and negative (with ciliates and no metal) controls were also set up as described above. An aliquot (1 ml) was taken out from each flask at 0 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h and 5 h after copper addition and metal uptake was determined as described above for each copper concentration. Using the same data, copper uptake rate during a specific period of time was also calculated in terms of copper taken up (ng) per unit time (min).

Analysis of gene expression at transcriptional level

The expression of *TfCuMT* at transcriptional level was studied in the absence or presence of different concentrations of Cu^{++} through Real Time PCR.

Effect of different copper ions concentrations on TfCuMT mRNA level

Three sets, each of four 100 ml conical flasks with 20 ml of modified Neff's medium were inoculated with 50 cells of *T. farahensis*. Three days after inoculation, three of the four flasks in each set were administered with 1, 5 and 10 µg/ml copper. The fourth flask in each set was used as negative control in which no metal was added. Fifteen min after copper addition, 1 mL aliquot of each culture was taken in eppendorf tube and centrifuged at 5800 xg for 10 min to pellet down the cells. The pellet was immediately used for RNA isolation through TRI reagent (Chomczynski and Sacchi, 1987). The isolated RNA (2 µg) was treated with *DNase*I before using it for cDNA synthesis with RevertAid™ M-MuLV Reverse Transcriptase (Fermentas cat # EP0442) according to Chaudhry and Shakoori (2010). The synthesized cDNA was subjected to Real Time PCR for quantification of mRNA levels in each sample (Weis *et al*., 1992).

Effect of single copper ion concentration for different time periods

Three 100 ml conical flasks with 20 ml of modified Neff's medium were inoculated with 50 cells of *T. farahensis*. Three days after inoculation, copper was added to the medium at 10 µg/ml. One ml culture was taken out at 0, 15, 30, 45, 60 and 120 min from each flask and used for isolation of RNA. *TfCuMT* mRNA level was quantitatively determined in each RNA sample through Real Time PCR as described earlier.

Real time PCR

Two sets of primers were used in real time PCR for quantitative analysis of *TfCuMT* (Table I). One set of primers was used to amplify a 120 bp fragment of *TfCuMT*. Second set was used to amplify a 97 bp fragment of 18S rRNA gene, a housekeeping gene, used as normalization factor.

Real time PCR was performed using 2x Maxima™ SYBR Green qPCR Master Mix (Fermentas Cat # K0221). The reaction mixture (20 µl) contained 1X sybr green master mix, 0.3 µM of each forward and reverse primer, 2 µl of 50X diluted cDNA. BioRad PCR plates (containing reaction mixtures) were short spun for 3-5sec. Real time PCR was performed in MyiQ2, BioRad. PCR reaction cycle comprised initial denaturation at 95 °C for 10 min followed by 40 repeats of denaturation at 95 °C for 25 sec, annealing at 56 °C for 25 sec and elongation at 72 °C for 25 sec with a final extension for 2 min at 72 °C. Melt curve analysis was performed between 70 °C to 85 °C with a gradient of 0.5 °C to check the specificity of amplifications.

To calculate the n-fold increase in mRNA level of *TfCuMT* in response to copper, normalized threshold value (ΔCt) of amplification curve of each sample was correlated to the ΔCt of sample obtained from culture with 0 mM copper (Pfaffle 2001). All calculations were done by iQ5 software of Bio Rad as well as manually using Microsoft Excel version 2010.

RESULTS

Growth characteristics of T. farahensis

Figure 1 shows the growth pattern of *T. farahensis* in Bold-basal salt, wheat grain and modified Neff's media. The ciliates showed lag phase for 24 h in all types of growth media, at the end of which they entered the log phase and reached their maximum, three days after inoculation. The overall growth pattern of organisms in all media remained nearly the same. However, growth maxima was 1.45 and 18.2 folds higher in Bold-basal and modified Neff's media as compared to wheat grain medium.

Table I.- Primers used in Real Time PCR studies of *TfCuMT* gene.

ID	Sequence (5' ---------- 3')	Target gene	Product Size	Reference
RT_F	5' TGATCCTTGCTCTTGTAACCC 3'	TfCuMT	120bp	This study
RT_R	5' CATTTGCAAGCAGAGGTCTT 3'			This study
NF_F2	5' TTCCGTTAACGAACGAGACC 3'	18S rRNA gene	97bp	Kutkienė *et al., 2*010
NF_R2	5' CTTCCATTGGCTTATTGCACA 3'			This study

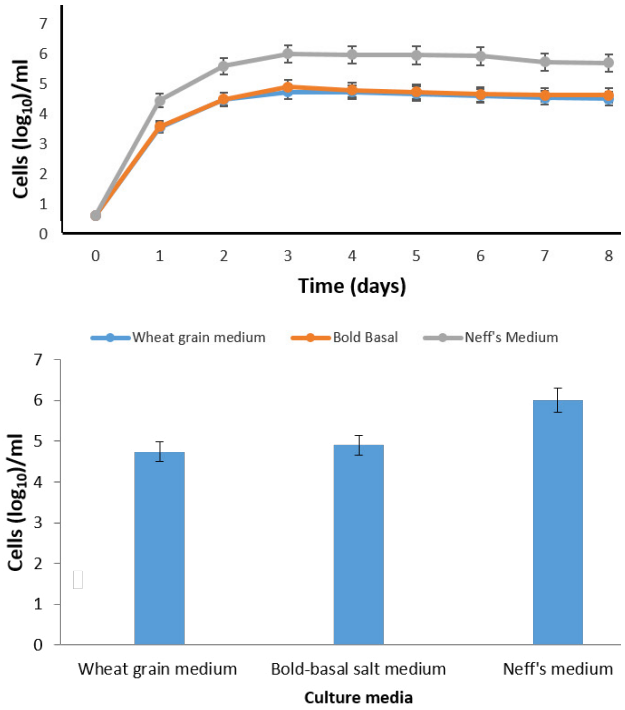

Fig. 1. Growth of *T. farahensis* in different media. In all media growth of *T. farahensis* was maximum after three days of inoculation. Organism showed 1.45 and 18.4 folds higher growth in Bold-basal salt medium and modified Neff's medium compared to wheat grain medium.

Optimum temperature for growth was observed 27±1 °C in all the three media *i.e.,* wheat grain, Bold-basal and modified Neff's media where total number of cells reached 5.5 x 10^4, 8.0 x 10^4 and 1.0 x 10^6 cells/ml, respectively (Fig. 2). Any change in temperature, whether raised or dropped, resulted in sharp fall in growth rate with 42 °C appearing to be the most adverse temperature for growth of *T. farahensis*. Effect of pH on the growth of *T. farahensis* is shown in Figure 3. Organisms were able to grow in a pH range of 6.0 to 8.0. *T. farahensis* showed optimum growth rate at pH 7.0 to 7.5 in all the three types of media. No growth was observed at pH 5.5 or pH 8.5.

The life span of *T. farahensis* was also observed in each of the three media (data not shown here). The organisms survived for more than six weeks in wheat grain and Bold-basal salt media while no organism survived after two weeks of inoculation in the case of modified Neff's medium. Thus comparative life span of *T. farahensis* population was 3 folds longer in the former case as compared to the later.

Effect of Cu++ on growth of T. farahensis

Under normal conditions, the organisms attained maximum growth in all media 3 days after inoculation. However in the presence of copper, the maximum growth was obtained 4 days after inoculation in wheat grain and Bold-basal salt media. No such delay was observed in case of modified Neff's medium under copper stress. In terms of cell density, 2.2 x 10^4, 3.5 x 10^4 and 0.2 x 10^6 cells/ml were observed at growth maxima in wheat grain, Bold-basal salt and modified Neff's media, respectively, which are 2.5, 2.3 and 5 times lesser than those attained under non stressed condition (Fig. 4).

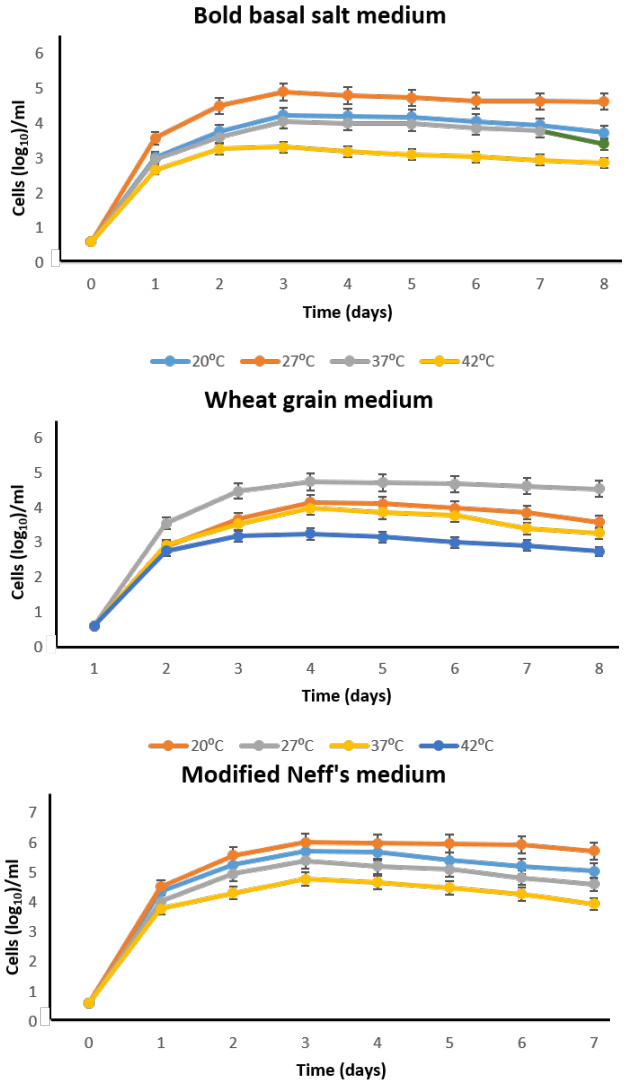

Fig. 2. Growth curves of *T. farahensis* at different temperatures. In all three different types of media, 27 °C is optimum temperature with the highest growth rate.

Metal stress also resulted in decreased life span of *T. farahensis*. The maximum resistance/tolerance dose of

copper ions in wheat grain, Bold-basal salt and modified Neff's media was 8 µg/ml (126 µM), 9 µg/ml (142 µM) and 80 µg/ml (1260 µM), respectively.

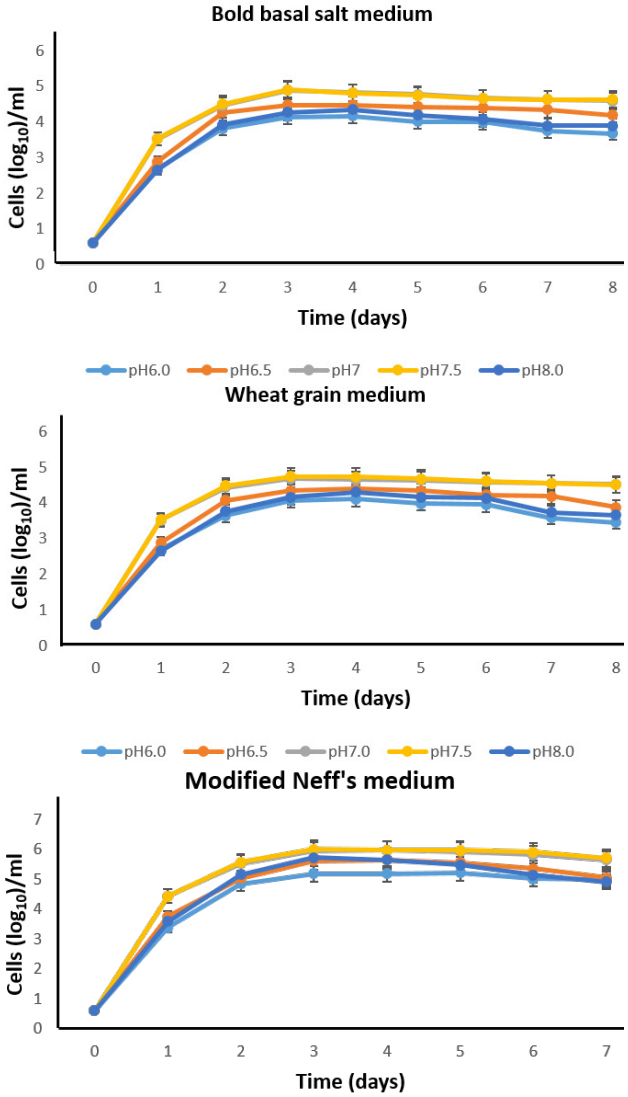

Fig. 3. Growth curves of *T. farahensis* depicting the effect of change in pH. Maximum growth was observed in all three media at pH 7.0 and 7.5.

Figure 4d depicts the growth of *T. farahensis* with different copper concentrations administered to ciliates on daily basis. It was observed that addition of copper at the rate of 1 and 5 µg/ml/day enhanced the growth enormously indicating its role as micronutrient. However, when copper was added at the rate of 10 µg/ml/day, the culture growth was retarded significantly, indicating copper toxicity when present in excessive amounts.

Cu uptake ability

Growing cultures of *T. farahensis* exposed to 30 µg/ml copper showed an increase in the copper uptake from the medium over a period of 96 h. Figure 5 shows the removal of copper ions from the medium by *T. farahensis*; 35.6 % of the metal from the medium after 24 h, 45.6 % after 48 h, 51.2 % after 72 h and 54.9 % after 96 h. The cells correspondingly showed concomitant increase in the concentration of Cu^{++}.

Effect of Cu^{++} concentration

Figure 6a shows copper uptake at different time intervals in modified Neff's medium supplemented with different concentrations of copper. Metal uptake appeared to be dependent on metal ion concentration in the medium as well as exposure time. Though, a gradual increase in copper stored in the cells was observed with increase in Cu^{++} concentration (5.56 and 9.78 µg Cu^{++}/ml in the presence of 10 and 50 µg Cu^{++}/ml in medium, respectively), but further increase in copper (100 µg/ml) in the medium lead to decrease in copper uptake (7.68 µg Cu^{++}/ml) within 5 h of copper administration.

Figure 6b showing copper uptake rate at various intervals of copper exposure depicts a bimodal copper uptake. At 5 and 10 µg Cu^{++}/ml, maximum uptake rate was observed during second quarter of the first hour, whereas in the case of 50 and 100 µg Cu^{++}/ml, the maximum uptake was recorded during the first quarter of the first hour, in each case, followed by a gradual decrease in the metal uptake, reaching approximately to zero at the end of second hour. Interestingly, Cu uptake was increased once again after 4 h of Cu administration in the case of 5, 10 and 50 µg Cu^{++}/ml, though the metal uptake did not reach the level observed during the 1st hour. Onset of this 2nd uptake was however, delayed with increase in copper stress. No bimodal uptake was observed in the presence of 100 µg Cu^{++}/ml probably due to cell death.

Quantitative expression of TfCuMT at transcriptional level

Effect of different Cu^{++} concentrations

Figure 7a shows the fold change in mRNA level of *TfCuMT* in response to different concentrations of copper in the medium. Expression of *TfCuMT* was proved to be copper dependent. A direct relationship was found between both as gradual increase in copper in the medium resulted in exponential rise in transcriptional level of *TfCuMT*. The mRNA level increased 30, 40 and 87 folds, respectively in response to copper concentrations at 1, 5 and 10 µg/ml.

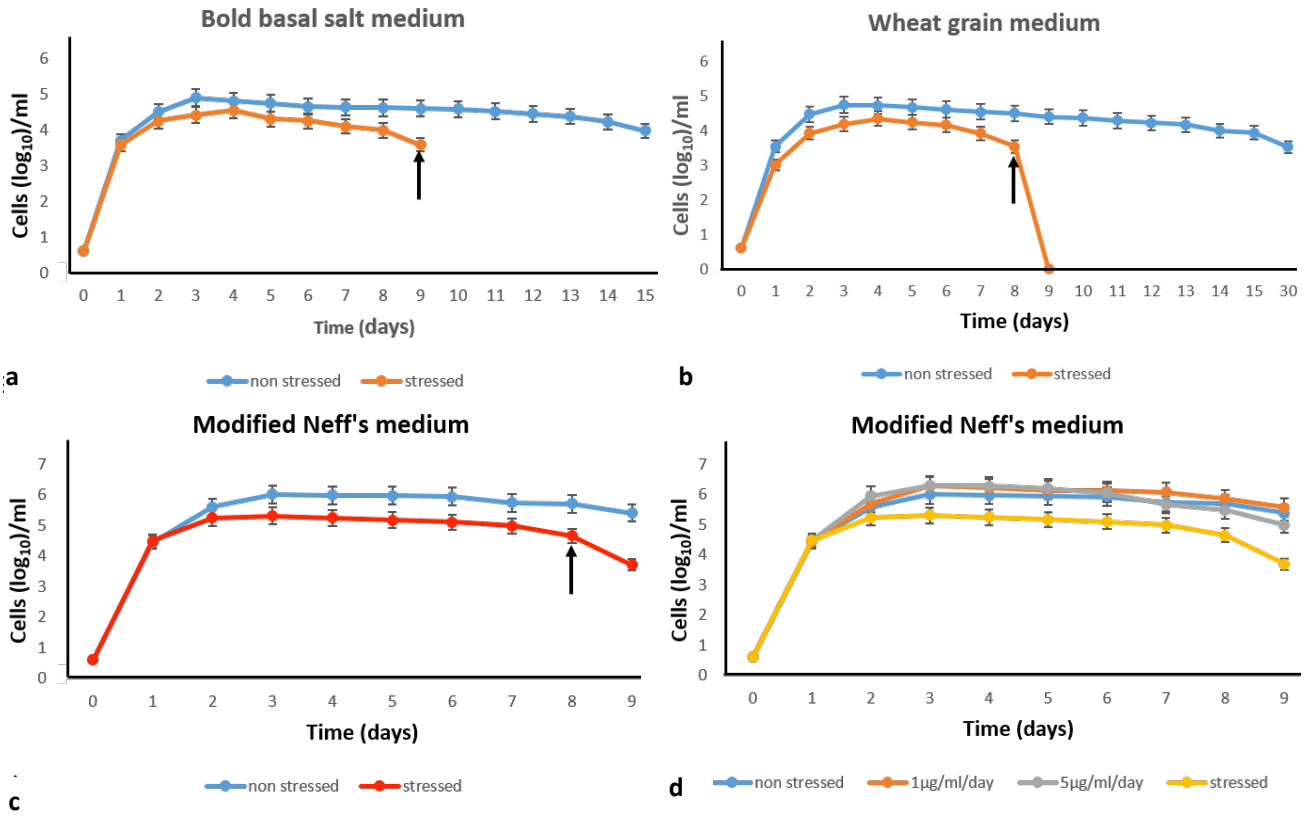

Fig. 4. Effect of copper stress on growth of *T. farahensis* in different media: a, 1 µg Cu^{++}/ml/day in Bold-basal medium; b, 1 µg Cu^{++}/ml/day in wheat grain medium; c, 10 µg Cu^{++}/ml/day in modified Neff's medium. Organisms showed an early death under stress conditions. Arrows indicate maximum resistance/tolerance dose of Cu^{++} for the cells; d, Comparative growth in modified Neff's medium in the presence of different concentrations of copper ions (1, 5 and 10 µg/ml/day) showed that low concentrations of copper (1 and 5 µg/ml) had a positive effect on growth rate of *T. farahensis*.

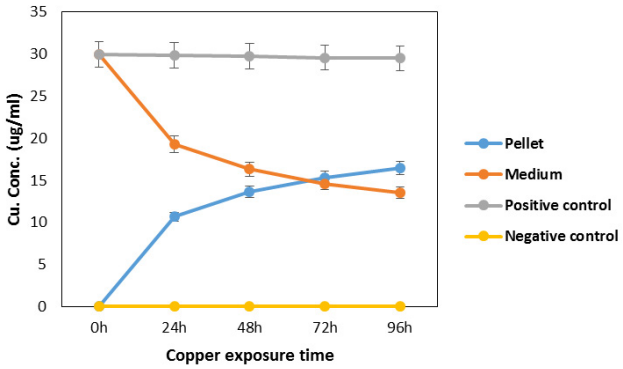

Fig. 5. Copper ions uptake by *T. farahensis* in modified Neff's medium with 30 µg/ml of copper. Organisms showed maximum uptake during first 24 h. A total of 54.9 % of copper ions were removed by the cells (pellet) from the medium during 96 h of metal exposure. A corresponding decrease in the concentration of Cu^{++} in the medium indicates that the metal has been taken up by the cells (pellet).

Effect of single copper ion concentration for different time periods

Figure 7b shows expression of *TfCuMT* in terms of mRNA levels in the ciliates growing in the medium containing 10 µg/ml of copper after every 15 min during the first hour and then at 120 min. The maximum mRNA level (87 folds of basal level) was observed at fifteen min after copper addition. However, the mRNA level decreased sharply within next 15 min (30 folds of basal level) followed by further decrease in the subsequent time intervals upto 14 folds of basal level at 120 min after Cu^{++} addition).

DISCUSSION

Heavy metals are known for their toxicity to the aquatic life ranging from planktonic to benthic and limnetic organisms (Monteiro *et al*., 1995). Most of the metal ions inactivate enzymes and proteins by binding with their thiol, amino and imino groups (Albergoni and Piccinni, 1983).

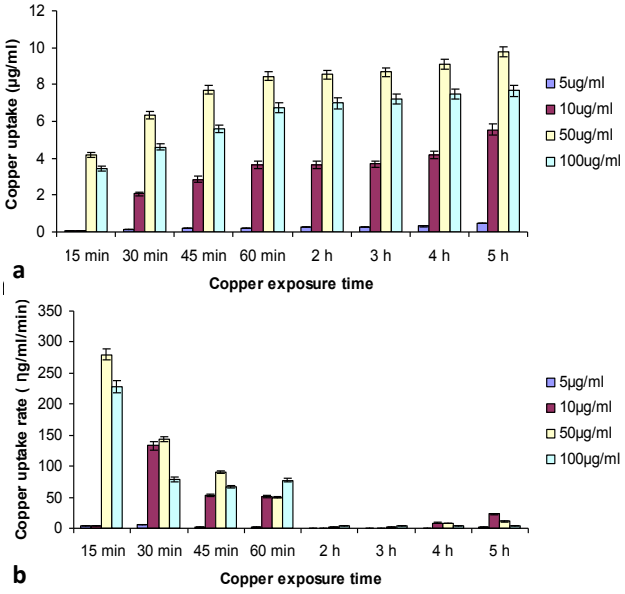

Fig. 6. Copper ions uptake by *T. farahensis* in modified Neff's medium containing different concentrations of metal ions at different time intervals. a, Maximum metal uptake was observed at 50 µg/ml stress level. At 100 µg/ml, there was a decrease in metal uptake, mainly due to decrease in viable count; b, In terms of metal uptake rate, a bimodal uptake of copper was observed at 5, 10 and 50 µg/ml copper ion concentration. The metal uptake rate was maximum during second quarter in the presence of lower (5 and 10 µg/ml) and during first quarter of first hour in the presence of higher (50 and 100 µg/ml) metal ion concentrations. The second cycle of metal uptake was initiated after 4 hours of copper addition. The metal uptake rate once decreased remained low in the case of 100 µg/ml metal concentration.

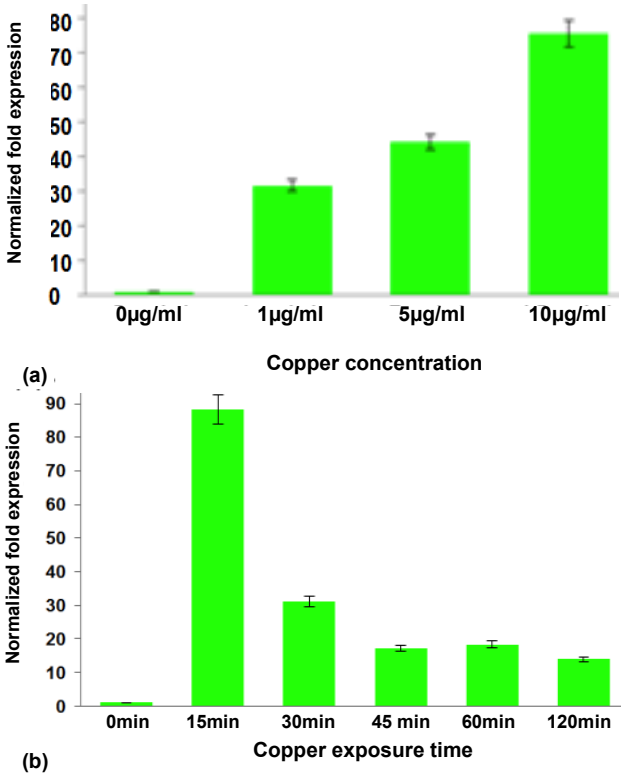

Fig. 7. Quantitative transcriptional analysis of *TfCuMT*. a, *T. farahensis* cultures induced with different copper concentrations, showed maximum expression at transcriptional level in the presence of 10 µg Cu^{++}/ml; b, Inducible transcriptional expression of *TfCuMT* at 10 µg/ml copper ion at different time intervals. Maximum mRNA level was observed 15 min after copper induction.

Heavy metal ions affect the survival of all types of organisms including protists by affecting their physiological processes like reduced endocytosis, decreased food uptake and inhibition of growth (Nilsson, 1981). Microscopic observation of different industrial wastewater samples showed the presence of different types of ciliates including *Paramecium*, *Plagiopyla, Stylonychia, Tetrahymena* and some algal and rotifers species. Microbes present in contaminated wastewater are of special interest due to their counter mechanism against heavy metals. Rehman *et al.* (2006, 2008) have reported the presence of ciliate *Euplotes mutabilis* in heavy metals contaminated wastewater ponds. Presence of ciliates in the heavy metal polluted wastewater indicates that they have adaptability to grow in polluted environment and they can be used to remove the metal ions from the polluted wastewater (Shakoori *et al.*, 2004; Rehman *et al.*, 2006).

T. farahensis pure culture was maintained in Bold-basal salt medium (Zahid *et al.*, 2014). Some contaminations observed along with *T. farahensis* were removed by using antibiotics, fungicides and keeping culture in dark. The low copper stress not only ruled out the contamination but also helped in selection of the copper resistant ciliates.

T. farahensis grown in modified Neff's medium showed higher growth rate than Bold-basal salt and wheat grain media. The difference in growth rate was mainly due to availability of nutrients and mode of nutrition. Higher growth rate in modified Neff's medium is due to plenty of nutrients (peptone, yeast extract, glucose and $FeCl_3$) in soluble form which are readily taken in by the process of phagocytosis while in Bold-basal salt and wheat grain media, *T. farahensis* have to prey on the bacteria to meet their nutritional requirements (bacterivorous mode of nutrition). Rehman *et al.* (2006) have reported the growth of metal resistant ciliates (*Euplotes*) in LB, molasses, wheat grain and Bold basal salt media. It was found that ciliates

cultured in Bold-basal salt medium showed maximum life span as compared to other growth media.

An inverse relationship between population growth rate and population life span was observed. In Bold-basal salt medium, *T. farahensis* showed lower population density (8.0 x 10^4 cells/ml) but culture was maintained for more than 6 weeks while in case of modified Neff's medium *T. farahensis* showed higher population density (1.0 x 10^6 cells/ml) but population was maintained for 2 weeks only. Decreased life span in case of modified Neff's medium was due to fast utilization of nutrients by higher population density. Moreover, nutrients were limited in this medium as compared to continuously growing bacteria in wheat grain medium.

Temperature affects the activities of cellular enzymes while pH causes a change in ionization of the biomolecules especially proteins which perform the cellular activities. *T. farahensis* showed growth in a broader range of temperature. They were capable of growing between 20 °C and 37 °C, with optimum temperature of 27±1 °C. This optimum temperature has been reported for different species of *Tetrahymena* (Dopheide *et al*., 2011). These results are also in agreement with those of Fields *et al.* (1984) who reported that the growth rate of *T. pyriformis* was maximum at 25 °C after three days of inoculation.

Effect of pH on the growth of *T. farahensis* showed that these organisms were able to grow in a broad range of pH. pH 7.0 to 7.5 was found optimum for the growth of *T. farahensis* while growth was inhibited below pH 6.0 and above pH 8.0. Weisse and Stadler (2006) have studied the effect of pH on the growth of three fresh water ciliates of genus *Urotricha* revealing that pH tolerance is mainly species specific. *U. furcata* tolerated a broad range of pH *i.e.,* pH 5.9-7.3 while for *U. farcta* and *U. castalia,* the tolerable pH was 4.4-5.3 and 6.8-7.9, respectively.

Copper ions have significant effect on growth of ciliates. Addition of 1 or 5 µg Cu^{++}/ml/day in modified Neff's medium causes a two times increase in growth rate. Increase in growth by adding low concentration of copper ions indicates its biological role as micronutrient for functioning of enzymes and other proteins. Nicolau *et al*. (2001) has also reported an increase in growth rate of protozoan community at low copper concentrations. However, abundance of same metal (addition of 10 µg Cu^{++}/ml/day) resulted in significantly decreased growth of the cells indicating its toxic effects. Rehman *et al*. (2009) have reported that excessive metal ions present in the growth medium resulted in a slower cell growth and delayed cell division. Higher copper stress resulted in a decrease of 92 % of *Euplotes* population after 22 days (Rehman *et al*., 2006). Copper toxicity is mainly due to generation of reactive oxidative species (ROS) which are toxic to biomolecules. Generation of ROS due to heavy metals have been studied in *T. thermophila* (Gallego *et al*., 2007) and Euglenoids (Watanabe and Suzuki 2002). Production of toxic radical species increased by increasing metals concentration and it was maximum near to LC_{50} (Gallego *et al*., 2007). The toxicity of different heavy metals vary for different ciliates but in general its order is Cu>Cd>Pb>Ni/Hg>Cr (Rehman *et al*., 2006).

The maximum resistance/tolerance dose for copper against *T. farahensis* is 9 µg/ml (142 µM), 8µg/ml (126 µM) and 80 µg/ml (1260µM) in Bold-basal salt, wheat grain and modified Neff's media, respectively. Higher tolerance in modified Neff's medium is due to protective role of organic rich medium against metal ions. Maximum tolerance against copper has been reported up to 220 µg/ml in *Vorticella microstoma* with the minimal survival of 44 days (Shakoori *et al*., 2004). The higher resistance against metal toxicity in some media is partially due to high concentration of organic molecules like peptone which have higher chelating capacity (Nicolau *et al*., 2001) as in the case of modified Neff's medium.

T. farahensis was capable of enduring metallic stress by virtue of different cellular mechanisms including bioaccumulation. It successfully removed 5.56, 9.78 and 7.68 µg Cu^{++}/ml culture within 5 h of 10, 50 and 100 µg/ml copper stress, respectively. It appeared the channels for Cu^{++} uptake were fully utilized when cells were exposed to 50 µg Cu^{++}/ml. Overall, there was an increase in the metal uptake by increasing metallic stress. However, at very high metal concentration, a decrease in metal uptake was observed, mainly due to the low number of viable count. In a similar study on soil ciliates, Diaz *et al*. (2006) have reported that increasing metal concentration increases the metal uptake capability of organisms. This bioaccumulation may be due to synthesis of MTs. Microorganisms have high tendency towards metal bioaccumulation (Harrison *et al*., 2006). They have higher surface to volume ratio, providing large surface area to contact with the polluted environment (Ledin, 2000). Among ciliates, *Euplotes* have been reported to remove 95 % of Cu^{++} within 96 h (Rehman *et al*., 2006).

T. farahensis showed maximum uptake within 15-30 min after copper administration which gradually decreased and again increased after 4 h. The shift in timings of copper uptake at higher concentration indicates that at higher metallic stress some rescue mechanism is activated which ensures the early synthesis of copper MTs to chelate the metal ions.

Quantitative expression of *TfCuMT* at transcriptional level was analyzed through real time PCR. There was an increase in *TfCuMT* transcripts by increasing copper stress. Various studies have reported that MT mRNA level

increases with an increase in metal induction. In case of *T. pyriformis* and *T. pigmentosa*, this increase was noted up to 5 µg/ml of cadmium (Santovito *et al.,* 2000). Induction of Cd and Cu resulted in 9 and 100 fold increase in expression of *T. thermophila* MTT2, respectively (Wang *et al*., 2011).

Copper stress boosted *TfCu*MT mRNA level to 87 folds within 15min, followed by a decreased level that remained 14 folds after 2 h of copper induction. These results are in close agreement with Boldrin *et al*. (2008) findings that MTs transcript level was maximum after 30 min of copper exposure. After maximum expression of MT, it is transiently down regulated but never becomes equal to basal level. Boldrin *et al*. (2006) have reported that copper treatment induced transcription of *T. thermophila* MTT2 followed by down regulation for several hours. The reduction in MT mRNA level is probably due to shortage of metal ions (Santovito *et al*., 2007). In *Drosophila*, MTs have been reported to inhibit their own expression through transcriptional factor inactivation (Egli *et al*., 2006).

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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