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Artificial neural network to predict the growth of *Leptospirillum ferrooxidans* in 9K defined medium

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Abstract

An artificial neural network (ANN) model was carried out to predict the cell concentration and ferrous iron concentration in the growth of strains of *Leptospirillum ferrooxidans* in batch culture, at different temperatures and pH values. A custom network with three interacting blocks was developed to carry out the prediction. The temperature, pH and time were fed as inputs to the network. In each block, a Multi Layer Perceptron was used and a tapped delay line was added in the first block. The appropriate architecture of the neural network model was determined through several steps of training and testing. The final ANN model was found to provide an efficient and a robust tool in predicting two variables simultaneously, in complex conditions such as non-linear and time-variant biological processes.

Keywords: Artificial neural network (ANN); prediction; *Leptospirillum ferrooxidans*; ferrous iron; custom network.

1. Introduction

The artificial neural network (ANN) is a non-linear estimation technique widely used in data processing and it has recently gained much importance because of its range of applicability in assessing the biological systems, which are time variant, non-linear and very complex (Basheer and Hajmeer 2000; Acharya et al. 2006). The ANN can be used in combination with statistics methods to process large data bases in situations where the relationships between the variables are complex and highly non-linear (Freeman and Skapura 1991; Haykin 1994). The statistical models require the verification of several assumptions, such as response's distribution, to guarantee the validity of the results in the inference process. In addition, there are many difficulties in applying the models for assessing complex systems with time variant, multiple variables, and non-linearity. In contrast, the ANN approach can detect the non-linear relationships in the data without the need for an explicit description of the complex nature of the underlying process in a mathematical form. The ANN needs an historical dataset that can be used to train the net. This dataset must be properly measured in the entry space and it must describe the phenomenon's fluctuations (Freeman and Skapura 1991).

The ANNs have found their applications in many aspects such as ecological and environmental sciences (Lek and Guegan 1999), prediction of the performance of wastewater treatment plant (Maged et al. 2004; Mjalli et al. 2007), modelling of river water quality (Singh et al. 2009), biotreatments for ions removal from aqueous solution (Prakash et al. 2008; Yetilmezsoy and Demirel 2008; Sahinkaya 2009), heavy metal sorption (Anagu et al, 2009); etc.

In biohydrometallurgy, some research studies about ANN applied to predict the growth of different microorganisms such as *A. ferrooxidans* (Laberge et al. 2000); *Leptospirillum ferriphilum* (Ozkaya et al. 2008; Nurmi et al. 2009) and *A. thiooxidans* (Liu et al. 2008) have been published. Additionally, Acharya et al (2006) have successfully predicted sulphur removal by *Acidithiobacillus* sp. To the best of our knowledge, current research has shown that there are no previous reports that have applied this methodology of modelling the kinetics of batch bacterial oxidation of ferrous iron using *Leptospirillum ferrooxidans*.

L. ferrooxidans is a chemolithotropic, aerobic, acidophilic, Fe(II) oxidizing bacteria that provides Fe(III) by the following equation:

$$2Fe^{2+} + 0.5\,O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$$

The ability of both *Acidithiobacillus ferrooxidans* and *L. ferrooxidans* to oxidize iron is widely known and the bioleaching of sulphide minerals is the most important application of that oxidation. In the bioleaching process, the dissolution of metal sulphide (MeS) in acidic solutions is improved in the presence of ferric ion (Falco et al. 2003). Due to

the biochemistry of the Fe(II), ion oxidation is different to that of *A. ferrooxidans L. ferrooxidans* is able to oxidize Fe(II) ions at very high redox potentials (Schippers 2007).

In this study, ANN was developed to predict the cell concentration (bact/mL) and Fe(II) concentration (g/L) using the temperature, pH values and time as input data during growth experiments of *L. ferrooxidans*.

The challenge in this work is the ability to the develop a network able to predict two values simultaneously: the cell concentration and the Fe(II) concentration. As the input information does not reflect the output variability (time variables for fixed temperature and pH values), the model needs to be more complex. Due to the fact that these input values have low quality information, the prediction task cannot be developed satisfactorily with a unique model of neural network. The alternative presented in this work consists of developing and implementing a model of a global net constituted by three modules or blocks jointly interacting to carry out the prediction task. To configure this kind of model a custom network was used. A custom network offers the necessary flexibility to use the output of a block as an input of the next one. In this way, the input information - that is insufficient initially - can be re-utilized to improve the net performance. The robustness of this model is evaluated by testing the net in new conditions, in which it has not been trained.

2. Materials and methods

2.1. Microorganisms

Six indigenous strains of *Leptospirillum ferrooxidans* used throughout this study were isolated from samples taken from La Silvita polymetallic sulphide mine, Neuquén, and from Rio Agrio and Lago Caviahue in the Geothermal Copahue-Caviahue (GCC) system, located in the north-west of Neuquén Province, Argentina (Chiacchiarini et al. 2009). The strains were identified as ''*L. ferrooxidans*-like'' bacteria based on the morphological characteristics of the cells observed with phase-contrast microscopy and scanning electron microscopy; and also on their physiology. Some of them were identified by 16S rRNA gene restriction enzyme analysis (Lavalle et al., 2008). The collection strain *L. ferrooxidans* DSMZ 2705 was also used as a reference strain.

2.2. Growing experiments

Growth and iron oxidation kinetics of indigenous strains and the culture collection were studied in shake flasks experiments with 9K medium (Silverman and Lundgren 1959). Trace elements solution (mg/L) $MnCl_2 .2 H_2O$, 62; $ZnCl_2$, 68; $CoCl_2 . 6 H_2O$, 64; H_3BO_3, 31; Na_2MoO_4, 10; $CuCl_2 .2 H_2O$, 67; acidified with 10N H_2SO_4 was added at a 1:1000 dilution in order to reach pH 1.8 in solution.

Experiments were carried out at different temperatures (20°C, 25°C and 30°C) and pH 1.4. The influence of pH on the growth was studied by adjusting the medium to different initial pH values (1.4, 1.8 and 2.2) using sulphuric acid. In these experiments the temperature was kept constant at 20°C, 25°C and 30°C for each respective experiment. The pH value was not controlled during the experiments. Samples were withdrawn at regular intervals to determine the cell concentration and Fe(II) concentration. The following initial conditions of cell concentration and Fe(II) concentration were approximately the same in all experiments $X_0 = 7.10^5 - 7.10^6$ bact/ml and $S_0 = 8\text{-}9$ g/L.

2.3. Analytical determinations

Ferrous iron concentration was followed by titration with a standard solution of potassium permanganate and the bacterial numbers (free in suspension) were quantified using a Petroff Hausser counting chamber and a phase-contrast microscopy.

2.4. Artificial neural network

2.4.1. Multi-layer feedforward perceptron

The multi-layer feed-forward perceptron (MLP) is a kind of ANN that predicts the value of one or more dependent variables, given the value of one or more independent variables. The MLP thus takes as input a vector X and generates an output vector (or scalar) Y, where Y is the prediction of the actual target T.

The MLP consists of three layers: a layer of input units that is connected to a layer of hidden units, which is further connected to a layer of output units. The number of neurons in the input layer is equal to the number of input parameters, and the output layer has a number of neurons corresponding to the parameters of prediction. Between them, there are one or

several hidden layers, which may contain zero or more neurons each. Additionally, every node of the hidden layer and the output layer has a constant input, the so called bias.

The activity of the input layer represents the raw information that is fed into the network. The activity of each hidden neuron is determined by the activities of the input neurons and the weights on the connections between the input and the hidden neurons. The behaviour of the output neurons depends on the activity of the hidden neurons and the weights between the hidden neurons. The weighted sums of the outputs from the input and the hidden layers are given by Eqs. (1) and (2), respectively:

$$N_j = f\left(\sum_{i=1}^{n} W_{ij}\, X_i^i + B_j\right) \quad (1)$$

$$Y_k = g\left(\sum_{j=1}^{m} W_{kj}\, N_j + b_k\right) \quad (2)$$

where, N_j is the output of j[th] hidden neuron, $W_{i,j}$ the weight between i[th] input neuron and j[th] hidden neuron, X_i^i the i[th] input of the input layer, B_j the bias of j[th] hidden neuron, n the total number of input neurons, Y_k the output of k[th] output neuron, $W_{k,j}$ the weight between j[th] hidden neurons and k[th] output neuron, bk the bias of k[th] output neuron, m the total number of hidden neurons, and *f* and *g* are the transfer functions.

The standard training algorithm is back-propagation -a generalization of the Least Mean Square algorithm-, and both (backpropagation and generalization) use mean square error as the same performance index. The algorithm is provided with a set of examples of proper network behavior: $\{X_1, T_1\}$, $\{X_2, T_2\}$, …, {Xn, Tn} (Hagan et al. 1996), where Xq is an input to the network and Tq is the corresponding target. As each input is applied to the network, the network output is compared to the target. The algorithm should adjust the network parameters (weights and bias) in order to minimize the mean square error:

$$MSE = \sum_{i=1}^{n} (T_i - Y_i)^2 \quad (3)$$

This iterative process continues until an error bench mark is satisfied. This bench mark must guarantee a reliable solution in the problem context.

In the gradient descent algorithm, the network weights are changed along the negative gradient of the performance function. The backpropagation (a gradient descent based method) or the Levenberg Marquand algorithms are often used as training procedures (Wieland and Mirschel 2008).

The adaptation of the weights during the training process can lead to a so called over training. This means that the neural network can reproduce the training data quite well but with the loss of ability to generalize. The phenomenon is especially important when only a few training patterns are available (Wieland and Mirschel 2008).

When the learning process is done, it is essential to evaluate the generalization capability of the net. It is necessary to have three sets of patterns. The training patterns, that usually have 80% of the total patterns, are used for training the net and to modify the weights and biases. Once the net is trained the validation stage begins. The net parameters are adjusted, mainly the number of hidden neurons and the transfer functions. In this stage, 10% approximately of the patterns are used. Finally, when the net is trained and validated, the testing stage is carried out. In this stage, approximately 10% of the data is used. This data is completely unknown to the net. The testing gives an idea of how the net behaves with new values. As a result, testing can be used to measure the reliability of the prediction. These sets are obtained from the available patterns and it is convenient that the separation is randomized.

2.4.2. Network architecture

In the present work, all input and output data were scaled so that they fall in the range [0, 1] using the following equation (4):

$$Pn = \frac{(p - \min p)}{(\max p - \min p)} \quad (4)$$

where *Pn* is the normalized parameter, *p* is the actual parameter, min *p* is a minimum of the actual parameters and max *p* is a maximum of the actual parameters.

Also, the data base was ordered according to decreasing values of Fe(II) concentration, so the net can learn form the historical data considering the involved chemical phenomena. Table 1 shows the values of pH and temperature used in the different stages of training.

Table 1: pH, temperature and number of patterns used in the different stages of training.

Stage	pH	Temperature (^{o}C)	Number of patterns	%
Train	1.4	25	70	
	1.4	30	43	
	2.2	25	63	
			176	**77**
Validation	1.4	25	10	
	1.4	30	18	
	2.2	25	8	
			36	**16**
Test	1.8	25	9	
	1.8	20	8	
			17	**7**

The strategy for designing the neural network involved starting with a 'simple' network structure and moving to more complicated ones. The first approach for the prediction task developed in this work consisted basically of implementing one MLP to predict the Fe(II) concentration and cell concentration at the same time. To do this several ANN configurations were developed. The number of neurons in the input layer were five: time (t), temperature (T), pH, Fe(II) concentration and cell concentration, and the output layer had two neurons, corresponding to the two parameters of prediction (Fe(II) concentration and cell concentration). Between them, there were one or several hidden layers, which may contain zero or more neurons, each (Figure 1).

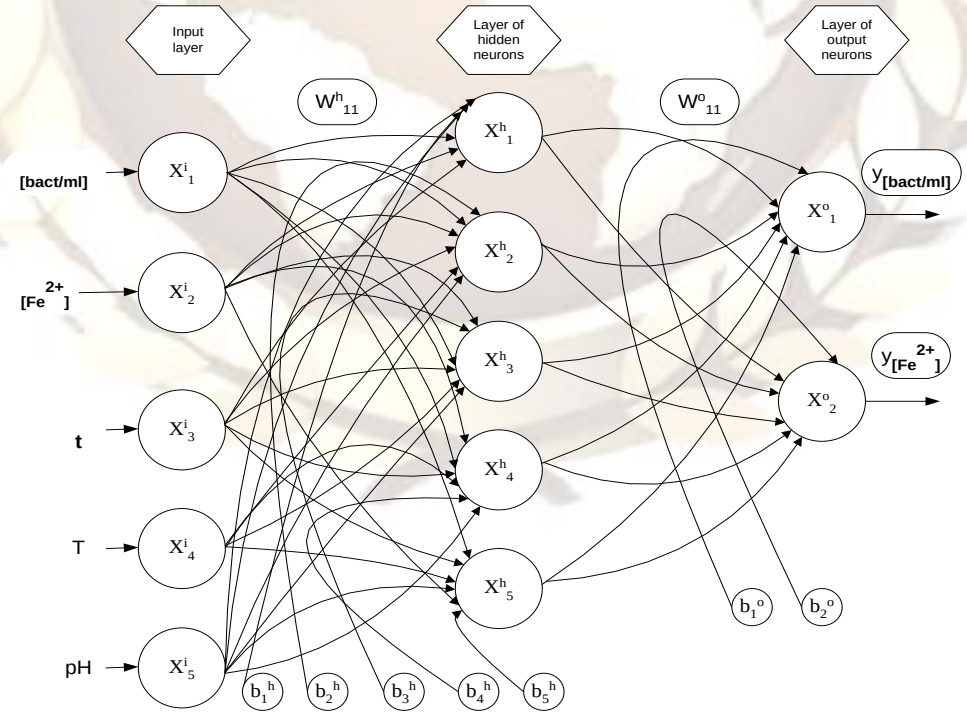

Figure 1: Neural network structure with one hidden layer for prediction of the cell concentration and ferrous iron concentration.

The number of hidden layers and the number of neurons in the layer was obtained by a trial and error method so as to minimize the error between the experimental and estimated results. The structure of the tested nets and their transfer functions are shown in Table 2. The output transfer function was always linear (LTF).

Table 2: Network structure and transfer functions tested in this study.

Number of hidden layers	Number of neurons in hidden layers	Transfer function
1	5	Sigmoid
1	8	Sigmoid
1	15	Sigmoid
2	5	Sigmoid and hyperbolic
2	8	Sigmoid and hyperbolic
2	15	Sigmoid and hyperbolic

The aim of this net is to have a structure that learns the general behaviour of the phenomenon in study. However, for the specific problem of prediction it is not possible to use this net because two of the input parameters have to be predicted. Once the initial network was trained and adjusted, the specific problem of predicting the output parameters for the new conditions of pH and temperature was faced. The inputs for this new network were time, pH and temperature, with the same two outputs. To do this, a custom network with three blocks was performed. A custom network is a flexible network object type that allows to define several architectures and to assign various algorithms (Hagan et al. 1996). In each block one of the outputs was predicted and then used as an input in the next block. Thus, an estimated value in one stage was then adjusted in the next one. This estimate-adjustment process was carried out with a block design in the global neural network.

It is necessary to emphasize the low quality of the input information to this net. Since for each condition of temperature and pH the only variable is the time, it is very difficult to achieve a good adjustment. In order to improve the results, a feed-forward input-delay back-propagation network was implemented in the first block. The tapped delay line (TDL) is connected to the network weight matrix through delays of 0 (actual value), 1 and 2 time units. The underlying concept behind the inclusion of a delay line in prediction's problems was discussed in Haykin (1994) and the implementation of this theory in practical problems was described in Hagan et al. (1996).

Finally, after many trial and error essays, and using the predictive capability of the MLP, a three blocks network with an input delay line was developed. A diagram of this global network is shown in Figure 2.

In Block 1, the input patterns are the values of pH, time and temperature. Each one of them enters in the net with their actual value and two step time delays. The aim of this block is to predict the value of Fe(II) that will be an input to the second block. The MLP used in this block has two layers, eight neurons in the hidden layer and Sigmoid and Linear transfer functions respectively.

Block 2 is a MLP network with two layers, five neurons in the hidden layer and Sigmoid and Linear transfer functions respectively. The inputs to this net are four: the same three input values of block 1 and the estimated Fe(II) concentration of block 1. The aim of this block is to improve the estimated value of Fe(II) and to make a first estimation of the cell concentration.

Block 3 has five inputs values: the same three input values of block 1 and the estimated values of Fe(II) and cell concentration in the block 2. The Fe(II) value was improved in the previous block and the same process is done with cell concentration. The input layer of the network in this block receives five input values, just as if it was an only network itself. But in the evaluation of the global three blocks network, the hidden relationships between the inputs patterns have the opportunity to be improved in the estimation-correction stages. In this block, the initial net with best performance found in the first approach was used. It has two layers, five neurons in the hidden layer and Sigmoid and Linear transfer functions respectively. The outputs of this block are the final estimated values of Fe(II) and cell concentration. The training algorithm used in the whole process is the Levenberg Marquardt. To evaluate the performance of the net the root of the mean square error (RMSE) was used in the different stages of training, validation and test. This parameter represents the deviations between the estimated and experimental results.

Additionally, the correlation between the experimental and the estimated values was calculated in different stages of training.

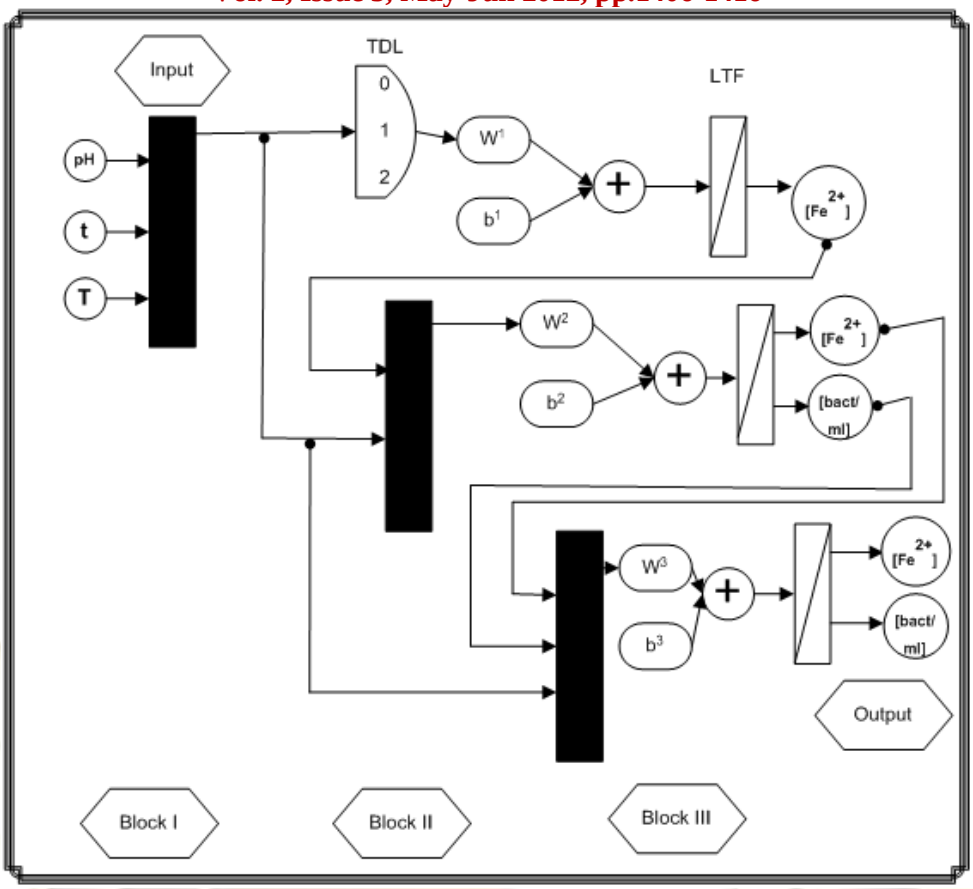

Figure 2: Global three blocks network diagram.

3. Results and discussion

The growth of some strains of *L. ferrooxidans* on 9K medium in shake flasks at different temperatures and pH values was studied. In a previous work, the specific growth rate μ was evaluated from the exponential phase of growing by linear regression. The results were similar to those reported by other researchers for other strains of *L. ferrooxidans* in batch cultures (Lavalle et al. 2008). The growth results were consistent with the ferrous iron oxidation rates obtained in the same conditions.

In order to predict the growth of *L. ferrooxidans* on 9K medium in a range of 20 to 30°C and pH 1.4 to 2.2, an ANN was proposed.

3.1. ANN Modelling

The first approach for the prediction task consisted the implementation of one MLP to predict the Fe(II) concentration and cell concentration at the same time. In order to obtain a good combination of the number of hidden layers and the numbers of neurons in those layers, a trial and error method was applied to minimize the error between the estimated and experimental results. Sigmoid and Hyperbolic transfer functions were used in the hidden layers and a linear transfer function in the output layer. The network was trained using the standard back-propagation algorithm, the gradient descent algorithm, in which the network weights are changed along the negative gradient of the performance function. Table 3 shows the results of the different configurations tested in the MLP.

The results reveal that the minimized RMSE (0.0027) was obtained when one hidden layer with five neurons was used. Sigmoid and Linear transfer functions were chosen for the hidden and output layers, respectively. This was the best performance network for adjusting the training values.

The next step in the study was to find a new network configuration that could predict Fe(II) concentration and cell concentration at different temperature and pH conditions, for which the net had not been trained. At the same time, this

network had to take advantage of the previous result. The strategy consisted of configuring a global network that contained specific blocks, each one of them dedicated to a specific task.

Table 3: RMSE of different MLP configurations.

Number of hidden layers	Number of neurons in hidden layers	RMSE
1	5	0.0027
1	8	0.0045
1	15	0.0122
2	5	0.0076
2	8	0.0985
2	15	0.2012

In the training stage, 174 patterns were used. The global net first block made a first estimation of the Fe(II) concentration. Since the input information consists only of temperature and pH conditions in time steps, a time delay line was used in the first layer. Although the input information was limited, the net's performance was satisfactory, obtaining a RMSE of 0.0998. The delay line used in this configuration provided this good result.

In the second bock of the global network an MLP was used to predict the Fe(II) concentration and cell concentration. The estimation of Fe(II) concentration was improved and the first estimation of the cell concentration obtained in this stage is not satisfactory yet. The global RMSE is 0.0494. The R value and the slope between the estimated and experimental patterns are 0.98964 and 0.98 respectively, indicating that this ANN model can estimate the Fe(II) concentration and cell concentration quite satisfactory in most of the cases (Figure 3).

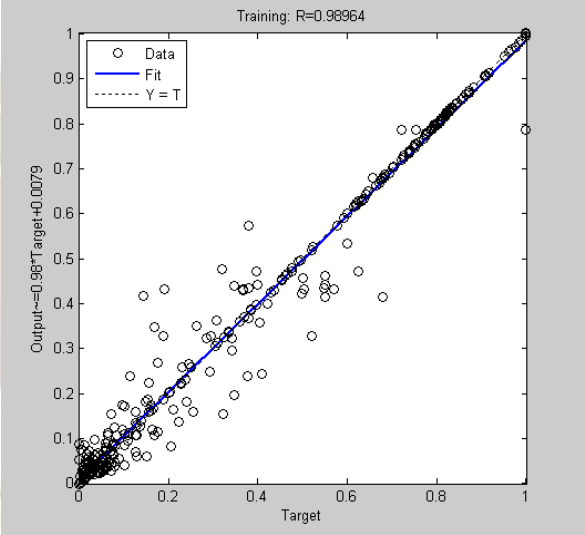

Figure 3: Linear regression between the network outputs and the corresponding targets in the training stage of the second block.

The final estimations of Fe(II) concentration and cell concentration were obtained in the third block of the global network. This network is able to estimate the values efficiently, with a RMSE of 0.0027. In this case the R value and the slope between the estimated and experimental patterns are 0.99997 and 1 respectively (Figure 4). The precise prediction of both values indicates that the ANN model used in this study is a powerful tool in predicting time-variant biological processes.

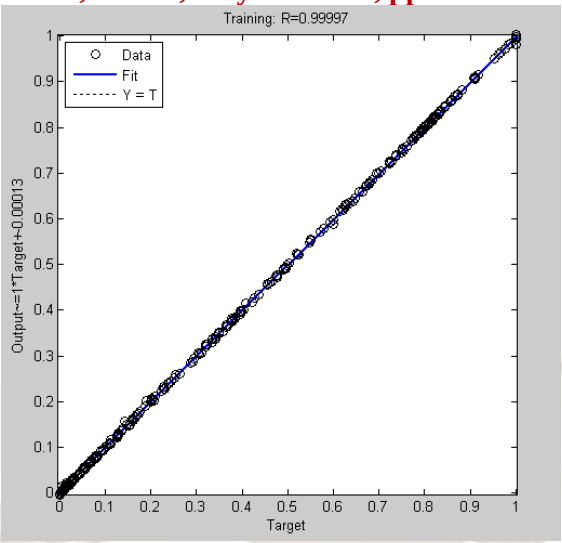

Figure 4: Linear regression between the network outputs and the corresponding targets in the training stage of the third block.

3.2. ANN Validation and test

In order to validate the ANN global model carried out in this study a set of 36 patterns was used. These patterns correspond to the growth kinetics carried out at pH 1.4 (25ºC and 30°C) and pH 2.2 at 25°C. The results of the RMSE for each block of the validation stage are presented in Table 4. The higher value of the RMSE in the second block is due to the first estimation of the cell concentration that was improved in the third block.

Table 4: RMSE of validation stage and testing stage.

Block	RMSE of validation stage	RMSE of testing stage	
		pH = 1.8 T = 25°C	pH = 1.8 T = 20°C
1	0.0972	0.1077	0.0698
2	0.1340	0.2102	0.1522
3	0.0067	0.0760	0.0845

Finally, once the global net was trained and validated, the testing set was simulated. This set was formed by 17 patterns, which were not used in the previous stages, i e, the measurements were carried out under different conditions: pH 1.8 at 25°C and 20°C. The results of the testing stage are presented in Figures 4. Their corresponding RMSE are also presented in Table 4. Both sets of data used in the testing stage correspond to specific growth kinetics. In this way, the number of patterns matches the evolution of time in the experimental test. Consequently, the output values were re-scaled and are shown in the original units of the predicted variables, i e, (g/L) for Fe(II) concentration and (bact/ml) for cell concentration.

It must be noted that the data used in the testing stage were completely unknown for the net. In the first case, pH and temperature values were among the corresponding values of training (Figure 5).

In the second case the temperature at which the data were taken (20°C) was outside the range of temperature of the training stage. Although the temperature was lower than the usual temperature range, it is a condition in which *L. ferrooxidans* is able to grow (Figure 6). This obligate acidophilic bacterium grows optimally in inorganic media within the pH range 1.3 to 2.0 (Coram and Rawlings 2002). The optimum growth temperature is between 28-30°C although it can grow across the range from 20°C to 40°C and the generation time increases up to 80 hours at temperatures lower than 20°C (Hallmann et al. 1993).

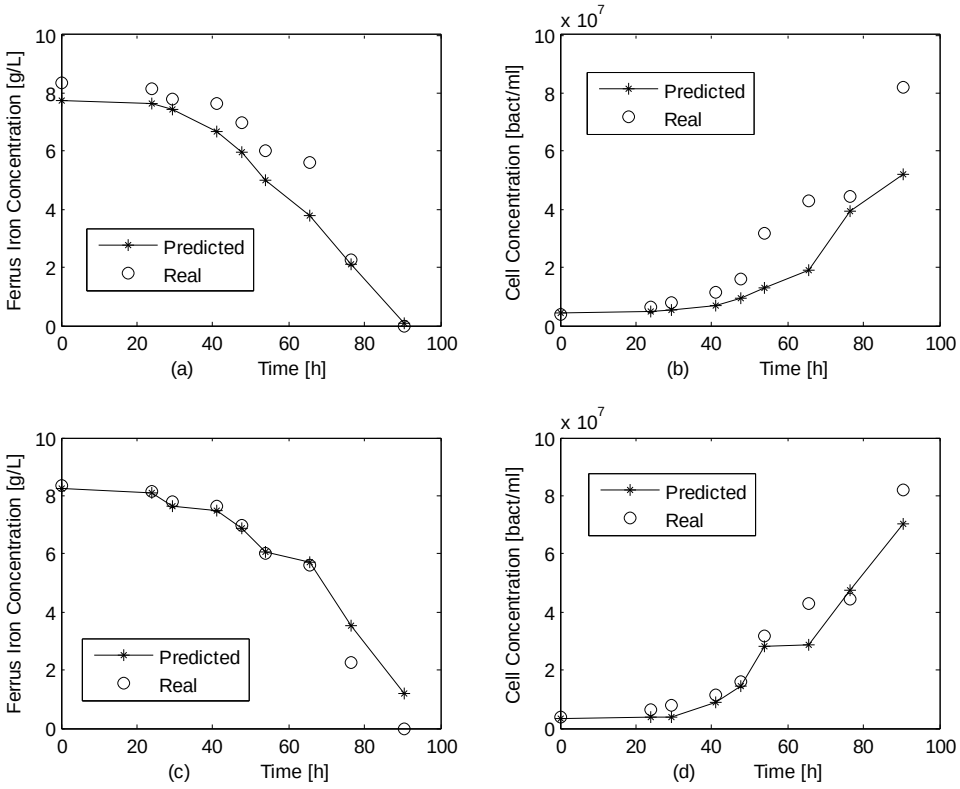

Figure 5: Predicted and measured ferrous iron concentration and cell concentration in the testing stage of the second block (a and b) and in the third block (c and d) at pH 1.8 and 25°C.

The resulting ANN model gives a satisfactory prediction of the cell concentration and Fe(II) concentration for the testing set. These results indicate that the global network can predict efficiently the Fe(II) concentration and cell concentration in experiments carried out under new conditions of pH and temperature.

4. Conclusion

This study presents a practical application to analyze one of the many different types of microbiological data. Considering the ductility of ANNs for building personalized architectures, a custom network was developed and implemented. The proposed architecture was a global net constituted by three modules interacting to carry out the prediction task. Each block's main purpose was to improve the prediction of the previous block. The configuration of each block in the global net was settled down through trial and error method. This allowed the configuration of the net with the number of layers and number of neurons in the layers to assure a minimum mean square error. Due to the low variability of the inputs of the first block, a delay line was used to re-utilize the input information and to improve the net performance. In the second and third block, an MLP was used and the Levenberg–Marquardt algorithm was selected to train the global net. The results show that the designed, trained and validated neural network model predicted the output variables in agreement with the real variables even though the data used in the testing stage were completely unknown to the net. Commonly, models based on multi-layer feed-forward perceptron networks predict only one output variable. A relevant aspect of the ANN model designed in this study is to simultaneously predict both, the oxidation of Fe(II) and cell concentration of L. ferrooxidans strains growing on 9K medium in batch culture. Although the problem of simultaneous prediction is not enough studied, the results obtained in this work using a global net are very encouraging.

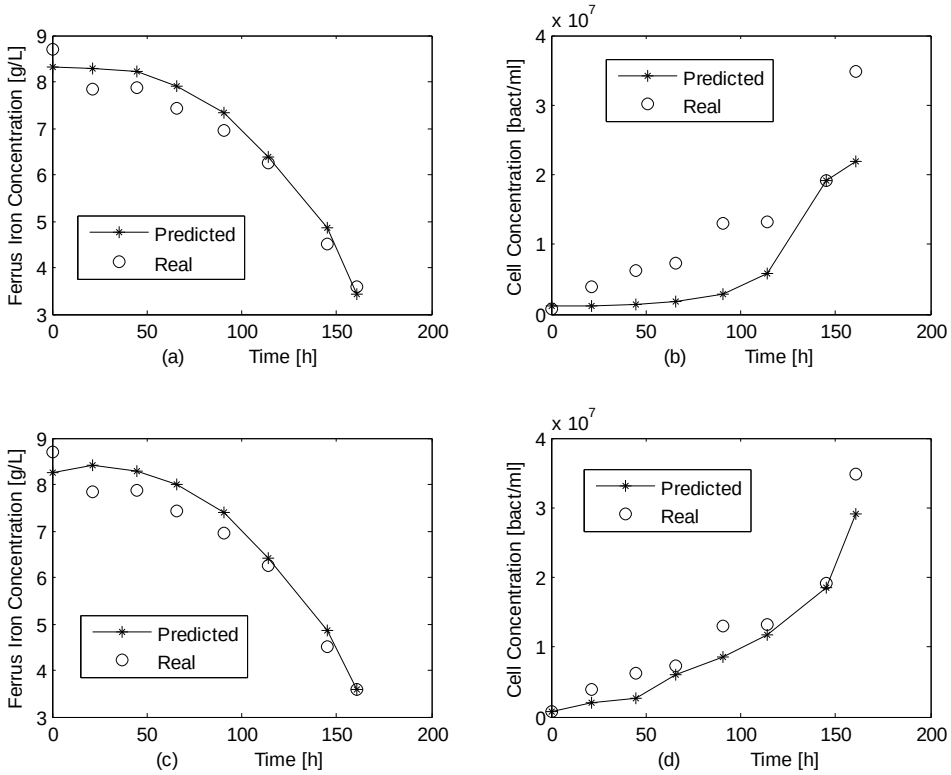

Figure 6: Predicted and measured ferrous iron concentration and cell concentration in the testing stage of the second block (a and b) and in the third block (c and d) at pH 1.8 and 20°C.

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