

## Fluorescence of pyoverdine synthesized by *Pseudomonas* under the effect of iron oxide nanoparticles

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### Abstract

The fluorescent emission of the thermolyzed *Pseudomonas aeruginosa* suspensions was measured in order to get a quantitative indicator upon the pyoverdine synthesis – as molecular basis of iron acquisition system of the bacterial cells. The influence of iron ions on the dynamics of pyoverdine was studied by supplying nano-sized iron oxide suspensions in the bacteria culture medium. The stimulatory effect of both iron oxide levels tested within this experiment (10 microl/l and 100 microl/l) was emphasized during the first 14 hours of incubation. The diminution of the pyoverdine synthesis was suggested for 20 and 22 incubation hours, but only in the case of the magnetite suspension highest level (of 100 microl/l). Potential applications to the experimental study of environmental pollution of the *Pseudomonas* species – known as having various ecological niches - with magnetic nanoparticles could be developed based on the results provided in this study.

**Key words:** magnetite colloidal suspension, fluorescent pigment, bacterial biosynthesis

### Introduction

The concern regarding the negative influence of nanosized particles upon the biotic and abiotic components of the biosphere was developed only in the last decades, when people could observe and measure their biological impact - though the natural metallic nanoparticles had existed in the environment since the beginning of Earth's history. The natural ubiquitous sources of magnetite fine grains are consistent with volcanic dust, most natural waters, soils and sediments, being generated by various geological and biological processes, while man made nanoparticles – either magnetic or non-magnetic materials – which are related to numerous technological processes are characterized by complex colloid and aggregation chemistry (SIMON & al. [1], GRIFFITT & al. [2]). Research data on magnetic nanoparticle biological effects showed their toxicity at the level of bacteria, algae, invertebrates and fish species, as well as mammals.

ZHU & al. [3] demonstrated that ZnO nanoparticles are very toxic to zebrafish embryos and larvae; HUND-RINKE & SIMON [4] evidenced that TiO<sub>2</sub> nanoparticles may exert ecotoxicological effects on algae and daphnids, which depend on the specific nanoparticle features; CUMBERLAND & LEAD [5] reported the influence of synthesized iron oxide nanoparticles to the aquatic environment; CHOI & al. [6] focused on the cytotoxicity of water-dispersible metal oxide nanoparticles in human tumoral cell lines (lung adenocarcinoma, breast cancer cells and glioblastoma cells), while LAI & al. [7] reported also the cytotoxicity of metallic oxide nanoparticles in human neural and non-neural cells. The biological influence of nanoparticulate metallic matter on the microorganisms was also investigated in the last years. Thus, a systematic approach to study and compare the *in vitro* cytotoxicity of selected engineered metal oxide nanoparticles in the test organisms (such as *E.*

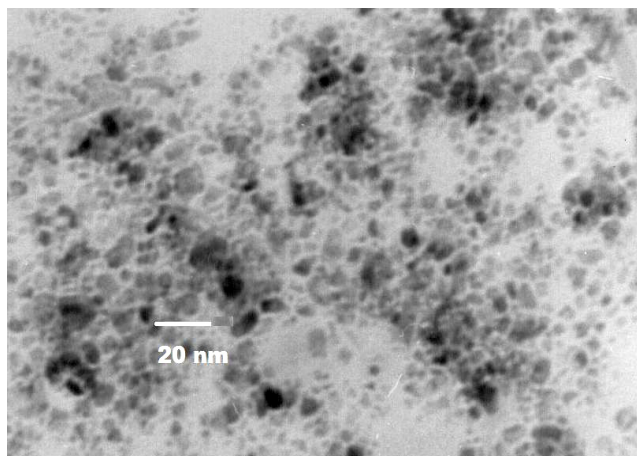
*coli*) was accomplished by Hu & al. [8], who found a high correlation between the cytotoxicity and the cation charges characteristic to the metal oxides, while WU & al. [9] studied the response of *E. coli*, *Mycobacterium smegmatis*, *Shewanella oneidensis* and *Saccharomyces cerevisiae* to TiO<sub>2</sub> and ZnO nanoparticles, by monitoring cell growth rates, morphological changes, key enzyme functions and gene expression.

The present experimental study was designed to provide an assessment of the magnetite nanoparticle effect in *Pseudomonas aeruginosa*, based on pyoverdine assay – the nanoparticle shell being different in comparison to that utilized in previous studies (POIATA & al. [10]), as well as the investigation method - fluorescence measurement – in comparison to turbidimetric investigation, used in other experiments by the authors of this paper (CREANGA & POIATA [11]).

## Materials and methods

**Biological material:** aliquots of 18 h aged cells of *Pseudomonas aeruginosa* ATCC test germ were inoculated in meat extract from OXOID, with 6.5 pH. Bacterial inoculum density at the wavelength of 560 nm was assayed using Meterteck spectral device and calibration curve, at 24 hours after the inoculation - during this time the temperature being monitored for all controls and test tubes ( $24.0 \pm 0.5$  °C). After the sample thermal treatment at 100.0 °C into STERICELL room and centrifugation for 15 minutes at 3.500 cycles/min, measurements on the supernatant were accomplished.

**Magnetite nanoparticles:** aqueous magnetite colloidal suspension was prepared according to MATEI & al. [12] by stabilizing magnetite (Fe<sub>3</sub>O<sub>4</sub>) - co-precipitated from ferrous and ferric salts - with tetramethyl ammonium hydroxide (TMA-OH). The average physical diameter of the obtained nanoparticles was of 7.94 nm as resulted from Transmission Electron Microscopy (TEM) data (Fig. 1), with a volume fraction of 2.05 % and a saturation magnetization of 123.30 Gs (RACUCIU & al. [13]). Colloidal iron supply into the microorganism culture medium was ensured by adding adequate aliquots of magnetic fluid dilutions – 10 microl/l and respectively 100 microl/l – achieving nanoparticles concentrations of  $10^{-15}$  -  $10^{-14}$  cm<sup>-3</sup>.



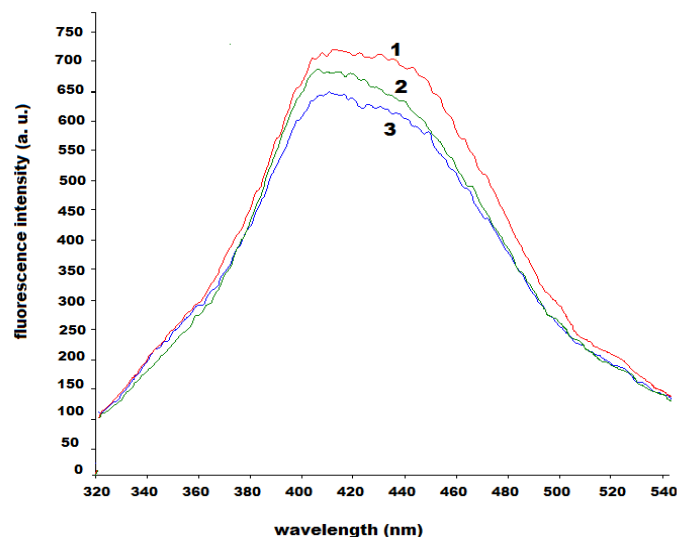
**Figure 1.** TEM image of aqueous suspension of Fe<sub>3</sub>O<sub>4</sub> core-TMA-OH shell nanoparticles

**Fluorescence investigation:** the assay of pyoverdine relative level was carried out after 2-4-6-8-14-20-22 hours of incubation at  $24.0 \pm 0.5$  °C with magnetic nanoparticle suspensions by fluorescence measurements upon the supernatant liquid containing bacterial pigment - released following the culture cell thermolysis (Perkin Elmer spectrofluorimeter, excitation light of 300 nm wavelength, fluorescence quenching being avoided by 1:10 dilution).

**Statistical analysis:** the experiment was repeated five times, the average values and standard deviation values being used for graphical plots. Statistical significance of the differences between control samples (without magnetic nanoparticle supply) and the test ones were discussed, according to *t*-test parameter.

## Results and discussion

Fluorescence emission, specific to the pyoverdine molecules (MEYER & ABDALLAH [14]), was characterized by a large band having the maximum at about 410 nm (Fig. 2).



**Figure 2.** The fluorescence bands of pyoverdine molecules released by *Pseudomonas aeruginosa* samples after 14 hours of incubation: 1) 100 microl/l magnetite colloidal suspension in the culture medium; 2) 10 microl/l magnetite colloidal suspension in the culture medium; 3) control sample (a. u. – arbitrary units)

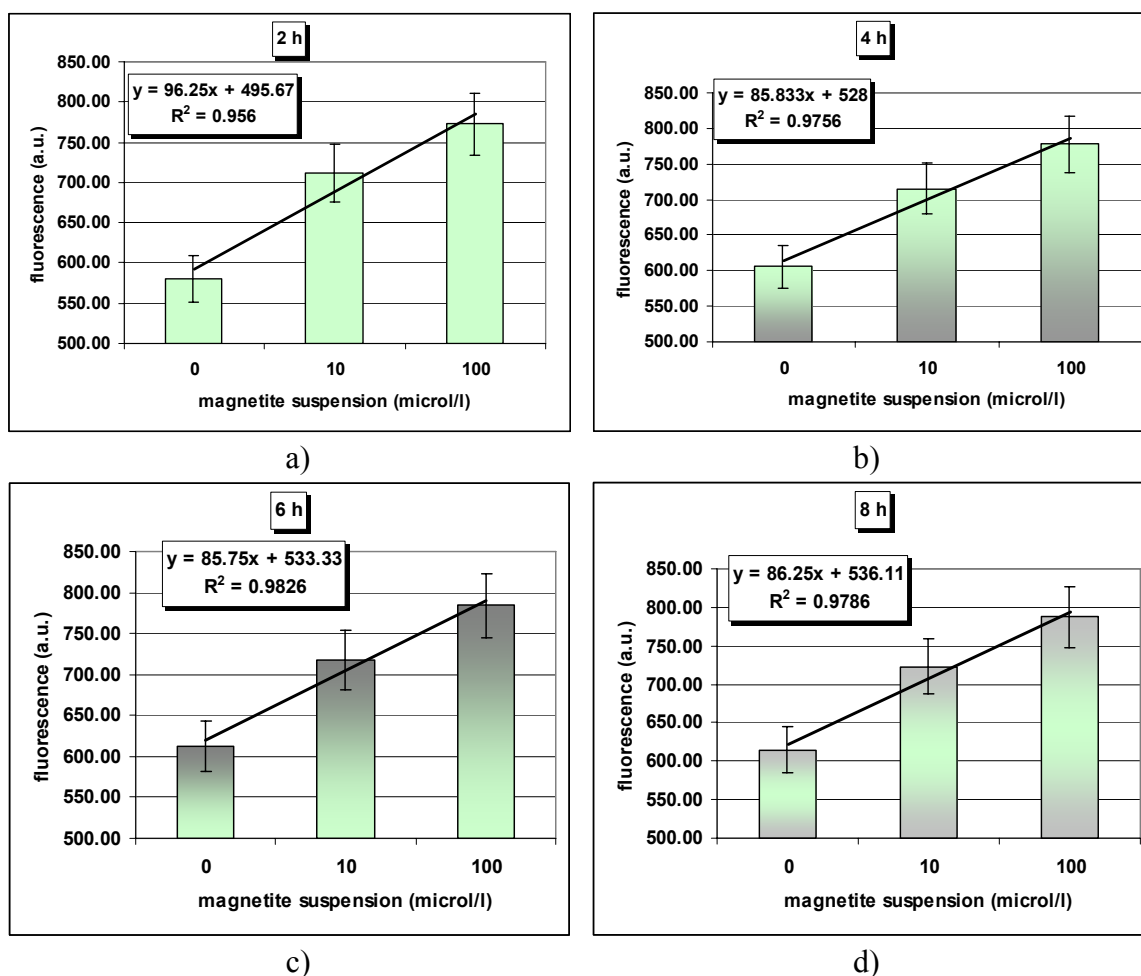
We mention that separate addition of magnetic fluid components (magnetite powder, tetramethyl ammonium hydroxide, water) did not result in detectable changes of fluorescent band, but the comparison with the previously investigation – carried out using magnetite particles coated with ammonia oleate [10] – revealed a shift of the fluorescence band maximum of about 80 nm towards lower wavelengths. This could be taken as an evidence that magnetite nanoparticles could be internalized by the bacterial cells only in the form of colloidal complexes (i.e. coated with adequate molecular shell) - I.J. SCHALK & al. [15]; also, the molecular electronic states of the pyoverdine released by the bacterial cells, were sensitive to the interaction with other molecular components of the supernatant, taken for quantitative analysis (remained in the culture medium after iron capture for pyoverdine synthesis).

**Table 1.** Linear approach of the fluorescence intensity dependence on the magnetite suspension concentration

Incubation time (h)	Line equation	Correlation coefficient	Incubation time (h)	Line equation	Correlation coefficient
2	$y = 96.25x + 495.67$	$R^2 = 0.956$	8	$y = 86.25x + 536.11$	$R^2 = 0.9786$
4	$y = 85.833x + 528.00$	$R^2 = 0.9756$	14	$y = 72.5x + 568.33$	$R^2 = 0.9996$
6	$y = 85.75x + 533.33$	$R^2 = 0.9826$	20	$y = 31.667x + 630.67$	$R^2 = 0.4390$
<i>t</i> -test	$P < 0.0001$		22	$y = 46.25x + 612.11$	$R^2 = 0.3264$

The results of the fluorescence measurements are presented in Fig. 3. In the first graphs, linear correlations, between the fluorescence intensity and magnetic nanoparticle level were evidenced, the bacteria sensitivity to iron loading being given by the line slope.

It is remarkable that the line slope (Table 1) is the highest for the samples analyzed after the first 2 hours of incubation: about 96 (Fig. 3 a) – in comparison to around 85 (Fig. 3 b, c and d) for the incubation durations of 4, 6 and 8 hours. Therefore, the influence of the magnetite on the pyoverdine biosynthesis was higher when the bacteria cultures were aged of about 2 hours (the fluorescence increase of about 33%) – all samples having initially the same cell density. Average standard deviation was of about 4%, while the statistical significance of the differences between the magnetite supplied samples and the control ones was assured according to the threshold of  $p < 0.0001$ .



**Figure 3 a-d.** The fluorescence dynamics in *Pseudomonas* samples for: a) 2 hours; b) 4 hours; c) 6 hours; d) 8 hours (a. u. – arbitrary units;  $R^2$ - the correlation coefficient)

The increase of the fluorescence relative intensity in the samples supplied with 100 microl/l magnetite suspension was smaller in the samples analyzed after 4, 6 and 8 hours of incubation (of about 28%), therefore one may conclude that the pyoverdine synthesis was still stimulated proportionally to the magnetite suspension concentration, though the bacteria sensitivity to magnetite appears to be lowered when the culture age increased.

In Fig. 3 e-g, the fluorescence variation in the samples analyzed after 14, 20 and 22 incubation hours is presented. The linear dependence between the fluorescence intensity – related to the pyoverdine level - and the concentration of the colloidal magnetite supplied in

the culture medium, were assured by good correlation coefficient ( $R^2 > 0.95$ ), only for the samples analyzed after 14 hours of incubation (Fig. 3 e), the line slope (equal to about 72) being significantly smaller than in the case of shorter incubation times (Table 1). The fluorescence relative intensity was increased with about 22%, in the sample supplied with 100 microl/l magnetite suspension, in comparison to the control sample. For the bacterial cultures aged of 20 and 22 hours (Fig. 3 f-g) the linear correlations could be established only formally since the correlation coefficient is small (under 0.5). Slight toxic effect of the highest magnetite suspension concentration (100 microl/l) is suggested since the fluorescence intensity was lower than that corresponding to the concentration of 10 microl/l (with about 5% and respectively 9%), however, in both magnetite supplied samples, the fluorescence intensity was higher than in the control samples (with 10% and respectively 14%).

In Fig. 3 h, the fluorescence dynamics evidenced atypical behavior of bacteria cell cultures for the relative long incubation times – where an increase (for 10 microl/l) or a diminution (for 100 microl/l) can be noticed, though logarithmic increase of sample fluorescence intensity could be established for the data corresponding to the first 14 hours of incubation, according to Table 2.

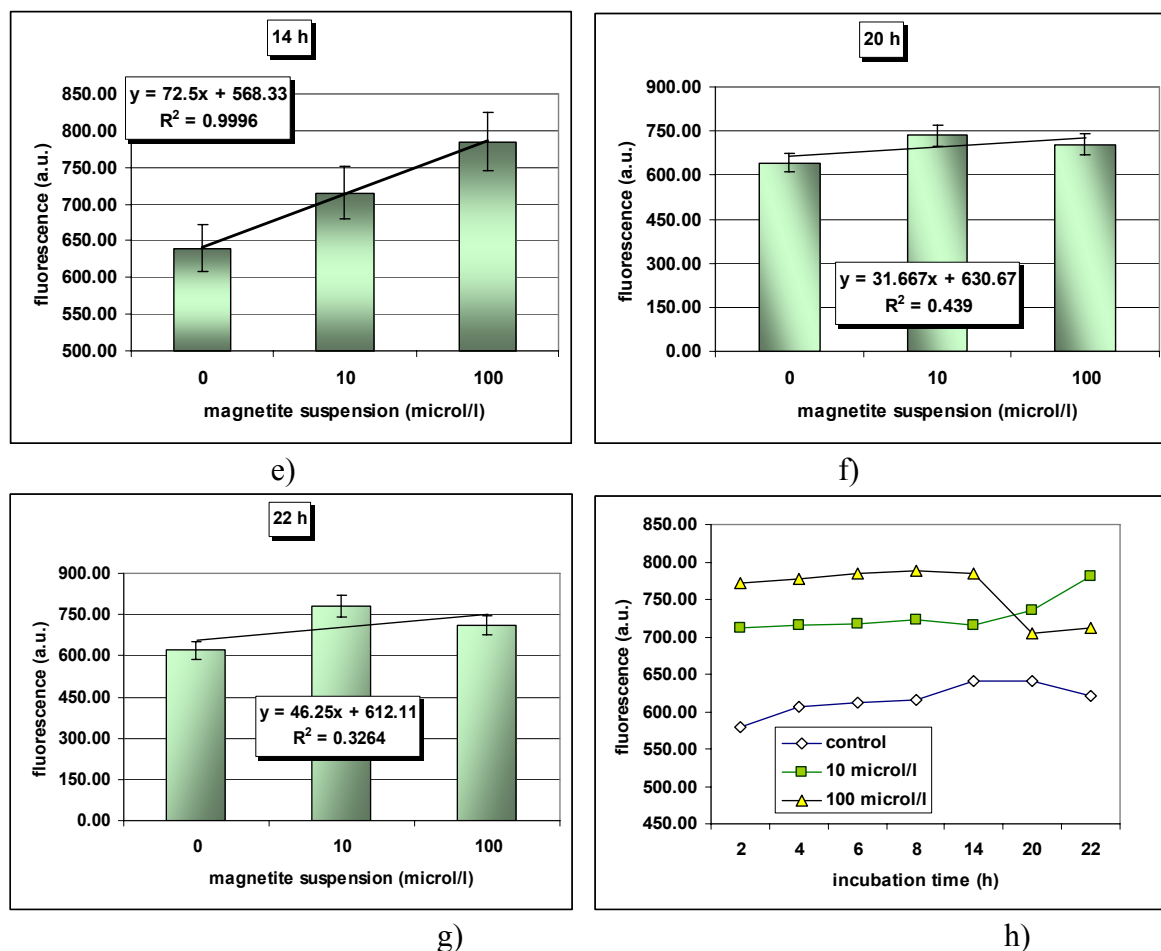
**Table 2.** The correlation between fluorescence intensity and the incubation time

Magnetic suspension level	Logarithmic approach	Correlation coefficient
Control	$y = 28.7660\ln(x) + 561.28$	$R^2 = 0.9620$
10 microl/l	$y = 7.5572\ln(x) + 705.92$	$R^2 = 0.9003$
100 microl/l	$y = 7.4935\ln(x) + 768.40$	$R^2 = 0.8026$

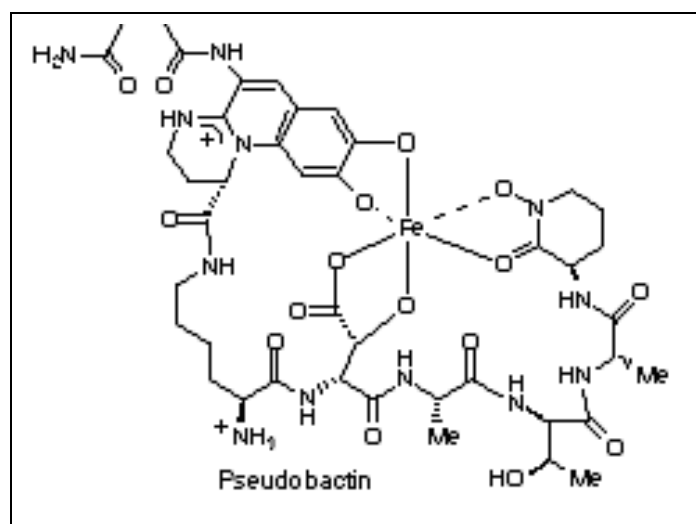
The fluorescence data can be interpreted as revealing the iron scavenger feature of *Pseudomonas* bacteria, resulting from the activation of the iron acquisition system based on the siderophore molecules (J.B. NEILANDS, [16]), only when the cellular stores are empty or contain little amount of iron, as well as when the iron amount in the culture medium seems to be lowering. For relative high amount of iron in the environment, the accumulation of pyoverdine tends to diminish after 20-22 hours of incubation, while for relative small amount of iron ions the tendency seems to be an increasing one – in contrast with the control samples where the logarithmic approximation can be extended inclusively on the data corresponding to 22 and 22 hours.

The pyoverdine (or pseudobactin) structure – an iron chelate belonging to the wide variety of bacterial siderophores, presented in Fig. 4, corresponds to a complex peptidic siderophore (blue greenish fluorescent pigment). However, siderophores can be biosynthesized by various other bacterial and fungal species - their nature as well as their iron uptake mechanisms being rather different. For instance, the ferrichrome siderophores of *E. coli* bacteria act in different way in comparison to the pyoverdine (pseudobactin) of *P. aeruginosa*, though similar six oxygen atoms systems are designed to couple an iron ion.

The peptidic complex can be exported by the bacteria toward the environmental medium, in order to capture iron ions, that can be further stored within the cell - as indispensable nutrient involved in the biosynthesis of peroxidase like enzymes and cytochromes. This system of iron acquisition was found to be benefic not only to the microorganism, but also to some plants that host *Pseudomonas* strains on their roots (BUYER & al. [17], MARSCHNER & al. [18]).



**Figure 3 e-h.** The fluorescence dynamics in *Pseudomonas* samples for: e) 14 hours; f) 20 hours; g) 22 hours; h) summary of fluorescence dynamics (a. u. – arbitrary units;  $R^2$  – the correlation coefficient).



**Figure 4.** Structure of *Pseudomonas aeruginosa* siderophore – the pyoverdine (pseudobactin)

The mission of the pyoverdine molecules to sequester traces of ferric ion ( $\text{Fe}^{3+}$ ) and to transport it through the cell membrane into the periplasmic space, (where the iron is set free by reduction to ferrous iron -  $\text{Fe}^{2+}$ ) requires "iron regulated outer membrane proteins" that assure the recognition at the cell surface and effectuate the transport [16]. Further investigations are expected to clarify the details of this ingenious cellular system, aiming to

identify suitable biotechnological applications, the spectral methods continuing to represent viable measurement techniques in molecular biology. The potential applications in the study of environment pollution with magnetite nanoparticles could be focused on the design of an iron biosensor system based on the *Pseudomonas* sensitivity to iron in the environment.

## Conclusion

The *Pseudomonas aeruginosa* sensitivity to magnetite colloidal suspension supplied in the culture medium was assessed by means of the line slopes approaching the correlations between the fluorescence intensity and the magnetite suspension concentration, the highest sensitivity being revealed for the cultures incubated for two hours with magnetite suspensions. The stimulatory effect of magnetite suspension on the synthesis of the fluorescent siderophore (pyoverdine or pseudobactin) was found during the first 14 incubation hours – the fluorescence intensity increasing, first with 33%, then with 28% and 22% (in the samples corresponding to 100 µmol/l in comparison to the control). Further, the iron scavenger characteristic of the studied bacteria cells could be noticed, since the fluorescence intensity dynamics revealed the continuous increase in the case of 10 µmol/l magnetite suspension level in the culture medium (relatively low iron ion concentration) while the fluorescence diminution tendency was found in the case of 100 µmol/l magnetite suspension (relatively high iron ions concentration).

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