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A novel plasma source for sterilization of living tissues

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Abstract. A source for the production of low-power plasmas at atmospheric pressure, to be used for the nondamaging sterilization of living tissues, is presented. The source, powered by radiofrequency and working with a helium flow, has a specific configuration, studied to prevent the formation of electric arcs dangerous to living matter. It is capable of killing different types of bacteria with a decimal reduction time of 1–2 min; on the contrary, human cells such as conjunctival fibroblasts were found to be almost unharmed by the plasma. A high concentration of OH radicals, likely to be the origin of the sterilizing effect, is detected through their UV emission lines. The effect of the UV and the OH radicals on the fibroblasts was analysed and no significant effects were detected.

Nonthermal, low-temperature plasmas, having populations of high-temperature electrons and ions at or near room temperature mixed with a neutral gas background, are emerging as a novel tool for *in vivo* nondamaging treatment of living tissues in biological and medical applications and, in particular, for nondamaging bio-sterilization [1, 2]. The use of plasmas produced with low-power sources for *in vivo* treatments has been pioneered by Stoeffels *et al* [3, 4], who have proposed it as an innovative approach to the treatment of dental cavities, which would not require drilling and the use of anaesthetics [5, 6].

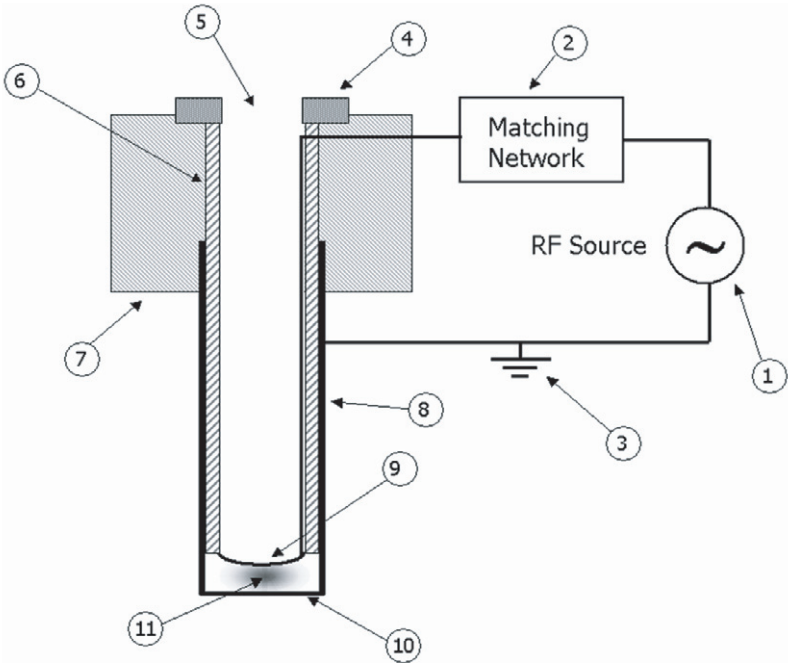

Figure 1. Scheme of the plasma source: (1) RF power generator, (2) matching network, (3) ground connection, (4) gas inlet, (5) He flow, (6) insulator tube, (7) support structure, (8) external electrode, (9) and (10) inner and outer grids, (11) plasma region.

In order to satisfy the requirement of plasma production without significant heating of the working gas, a new generation of plasma sources specifically designed for this kind of application is being developed [3, 7, 8]. In this paper, we describe a novel plasma source operating at radiofrequency (RF) specifically designed for producing a low-temperature atmospheric pressure plasma to be used for non-damaging sterilization of living tissues.

The device, schematically shown in figure 1, consists of two coaxial tubes: an external one made of copper, 80 mm long, with an outer diameter of 12 mm, and an internal one made of an insulating material (polyethylene, 100 mm long, 10 mm outer diameter). Each tube is closed at one end by a brass grid, made of wires with 0.2 mm diameter spaced by 1 mm. The external copper tube is electrically connected to the external brass grid. The tubes can be moved with respect to each other using a micrometric screw, so as to adjust the distance between the two grids to a value that allows the breakdown but prevents arc formation ($\sim$1 mm). In particular, the external electrode is grounded, so that the possibility of arc formation between the electrodes and the substrate to be treated is totally avoided. This is a basic requirement when working with living matter, as arcs concentrate power on a very small area, seriously damaging the substrate. The working gas is helium of 99.9999% purity, which is injected into the inner tube. Typical gas flows are in the range 0.5–5 litres min^{-1}, with 1.75 litres min^{-1} identified as a good value for stable plasma formation with limited gas consumption. It is worth mentioning that plasma start-up is helped by initially setting a high flow value, which can then be reduced to the desired one. The power supply used for our prototype is a radio transmitter operating at fixed power (5 W) and adjustable frequency. The transmitter is coupled to the device by means of a minimal matching network realized by a series 100 μH inductor; the combination of this inductor and

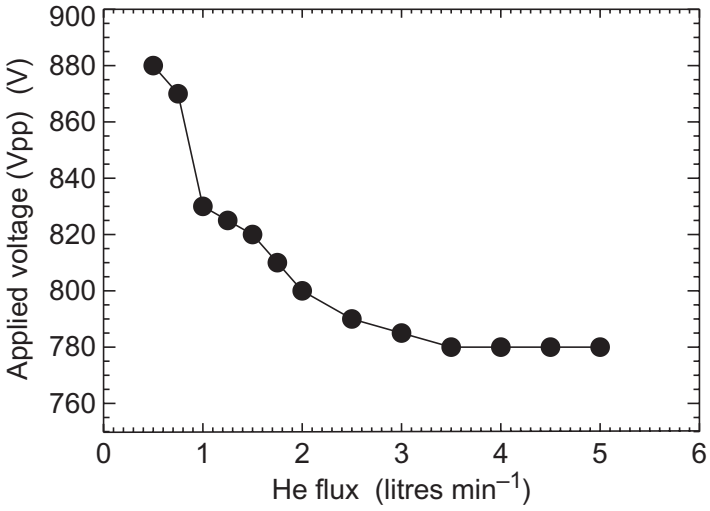

Figure 2. Voltage measured between the grids plotted as a function of the He flux, for a given power level (5 W) sustained by the radio transmitter. Peak-to-peak values of the sinusoidal waveform are shown.

the parasitic capacitance of the device, estimated to be around 10 pF, gives rise to a resonance around a frequency of 4.8 MHz, which yields a voltage up to 900 Vpp on the plasma. It is found that the resulting voltage applied to the electrodes depends on the He flux, as shown in figure 2. The arrangement is far from being optimal in terms of reflected to transmitted power ratio, which is larger than 2. While a more sophisticated set-up could be easily realized, we found that in this way an appropriate power level could be coupled to the plasma source. In fact, most of the transmitted power is dissipated on the inductor, and we estimated that less than 1 W is actually coupled to the plasma itself, so that no appreciable heating of the treated substrate has been measured.

The sterilizing effect of the plasma has been tested by treating different types of bacteria cultures, namely, in order of resistance, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Bacteria have been grown in the proper medium at 37 °C, and then on the day of the experiment 500 μl of the culture were transferred to 5 ml of fresh medium and incubated with shaking until the optical density reached 0.1 at 590 nm. Bacteria were then serially diluted to obtain suspension concentrations of 10^6 colony formation units (CFU) per ml. Afterwards, 30 μl of the solutions containing various amounts of microbes were placed in 24-well cell culture plates of tissue culture (NUNC, Roskilde, Denmark; growth area 2 cm^2 and well volume 3.5 ml), 15 mm in diameter. The treatment was performed by positioning the plasma source external grid 1–2 mm above the bacteria cultures for the chosen treatment time interval (from 30 s to 5 min). After treatment, each sample was collected and vital microbial count was performed by seeding the sample on the proper agar medium and incubating at 37 °C for 16 h. The number of CFU appearing on each culture dish was counted to assess the number of surviving organisms. As figure 3 shows, different bacteria have different fractions of surviving CFU, but, in all cases, an exponential decay is found. The decimal reduction times are 0.97 min for *E. coli*, 0.87 min for *S. aureus* and 2.38 min for *P. aeruginosa*.

The sterilizing effect of the source is mainly due to atomic and molecular radicals, produced by influence of plasma high temperature electrons on oxygen, nitrogen and water molecules present in the helium flow. In particular, oxygen free radicals, like atomic oxygen O

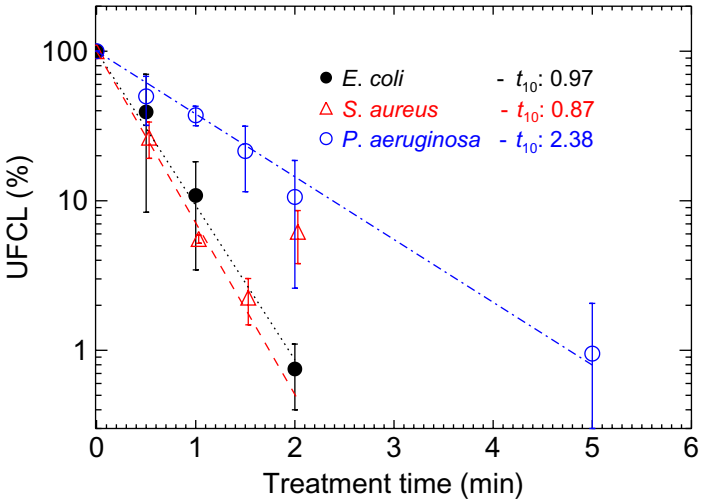

Figure 3. Colony number counts after plasma treatment for different bacterial strains, plotted as a function of the treatment duration. The straight lines show the results of exponential fits. The resulting decimal reduction times are reported in the figure.

and hydroxyl group OH, and other reactive oxygen species (ROS) have an essential role in the plasma germicidal effect [9, 10].

The presence of active radicals and UV radiation in the source was tested by means of spectral measurements. The plasma radiation has been detected by a Mini-spectrometer (Hamamatsu C10082 CA), integrated with a back-thinned type linear CCD image sensor (2048 pixels). The spectrometer measures within a range from 200 nm up to 830 nm with a single spectrum. A quartz optic fibre of 1 mm diameter core and 100 cm long, matched with the spectrometer, has been located in front of the plasma at a distance of 2 mm. Two typical spectra recorded with a He flux of 1.75 litres min^{-1} and of 4.5 litres min^{-1} at room pressure are shown, as they appear without the absolute calibration correction, in figure 4. The spectra display the presence of the N_2 molecule emission lines with their vibrational band, together with a high concentration of OH radicals (308 nm), with most of the lines concentrated in the UV region. The intensity of some selected emission lines (obtained with a Gaussian fit) is observed to depend on the He gas flow, as shown in figure 5. In the experimental condition corresponding to a gas flow of 0.5 litres min^{-1} the intensity of the selected lines is low due to ionization, which is occurring in a small volume of the inter-electrode region. This is simply deduced by visual inspection showing strongly localized emissive plasma spots, which indicate that the plasma is formed only in some regions, probably related to imperfect planarity of the grids. For He fluxes above 0.75 litres min^{-1}, corresponding to diffuse plasma generation, a decrease of the intensity of N_2 is observed with the increase of the He flux, along with an increase of the intensity of the OH radicals line and a slight increase of the He I. As no absolute calibration correction has been taken into account, no absolute comparison between different line intensities is reliable, and hence no information about the relative species concentration can be deduced. Due to a drop of spectrometer sensitivity in the wavelength range around 700 nm, the He I line intensity is particularly low; for this reason it has been multiplied by ten in the figure.

The consequences of the plasma interaction with tissue cells were studied using conjunctival fibroblasts seeded in 12-well cell culture plates (NUNC, Roskilde, Denmark;

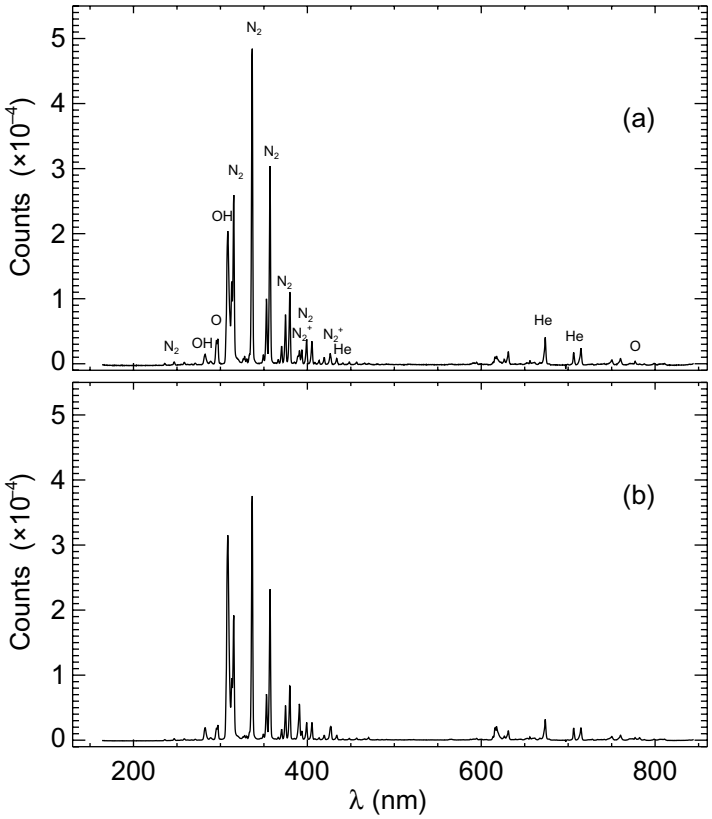

Figure 4. Emission spectra of the plasma, showing a high radiation intensity in the UV range. The different spectral lines are marked according to their origin: (a) spectrum taken with a He flux of 1.75 litres min^{-1}; (b) spectrum taken with a He flux of 4.5 litres min^{-1}.

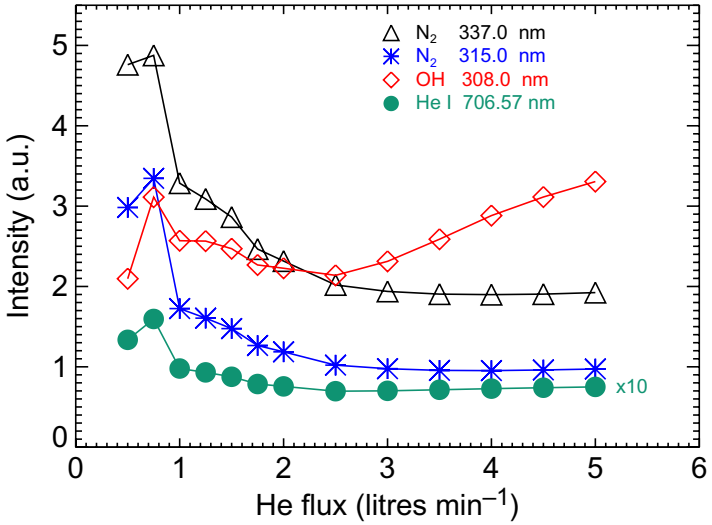

Figure 5. Intensity of different emission lines plotted as a function of the He flux. The He I intensity is multiplied by 10.

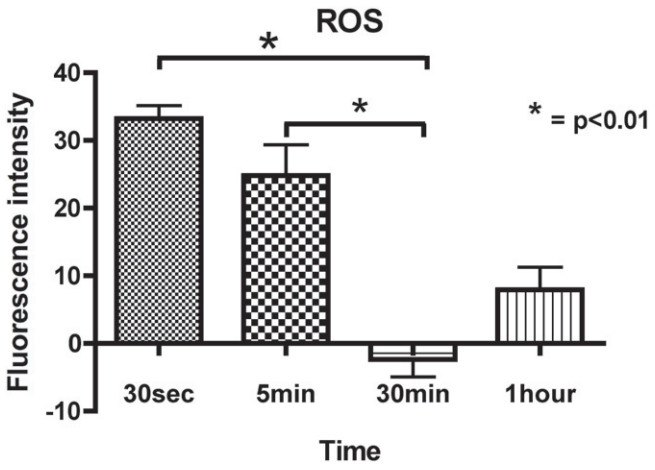

Figure 6. Dynamics of ROS level in conjunctival fibroblast cultures after 2 min of plasma treatment. The fluorescence values in the control samples have been subtracted from those in the test samples.

growth area 3.8 cm^2 and well volume 6 ml) and cultivated in the presence of 2 ml of Ham's F12 medium (Sigma, Milan, Italy) supplemented with 20% foetal calf serum and antibiotics (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM l^{-1} L-glutamine), at 37 °C in 5% carbon dioxide in a humidified atmosphere. Before plasma treatment, culture medium was discharged and cell layers were covered by 300 μl of fresh medium, so as to prevent their dehydration. The plasma source was placed 1–2 mm above the culture medium surface.

In a living organism, the excess of ROS entails an imbalance in cellular homeostatic mechanisms, called oxidative stress. To estimate the intracellular oxidative level in the conjunctival fibroblast cultures, after plasma treatment, a fluorigenic probe, the 2′, 7′-dichlorodihydrofluorescein (H_2DCFDA), was used. The acetate group of this compound is cleaved by cytosolic esterases to yield DCFH, a polar nonfluorescent molecule retained by the cell. Cytosolic oxidants convert DCFH to its fluorescent derivative, 2′, 7′-dichlorofluorescein [11]. Accumulation of oxidized DCF was measured with a 480 nm low-pass excitation filter and a 520 nm high-pass emissions filter. As shown in figure 6, fluorescence emission was measured at different time points, after 2 min plasma treatment. The maximum emission detection was relieved after 30 s from treatment; subsequently, the emission was reduced to 0 in 30 min. This means that fibroblasts react well against ROS attack: they can quickly activate anti-oxidative systems.

Even if a low-power, low-temperature plasma is not a very effective source of high-energy UV emission [12], it could in principle cause DNA damage, responsible for cytotoxicity and mutagenicity in living cells. Among specific UV effects on cells there is the formation of thymine dimers (TD) in DNA strands that inhibit the ability of cells to replicate properly. For this reason, the presence of TD in DNA of cells after a 2 min plasma treatment was investigated by immunofluorescence microscopy using a monoclonal antibody raised against TD [13]. No TD was detected.

The global effect of the source on living fibroblast cells has also been tested, to prove that the nondamaging disinfection selectively kills the pathogens but not the tissue cells. Effects on cell viability were assed by MTT test [14], performed 15 min after treatment of two-dimensional cell cultures for the same time interval (from 30 s to 5 min) used for bacteria, at

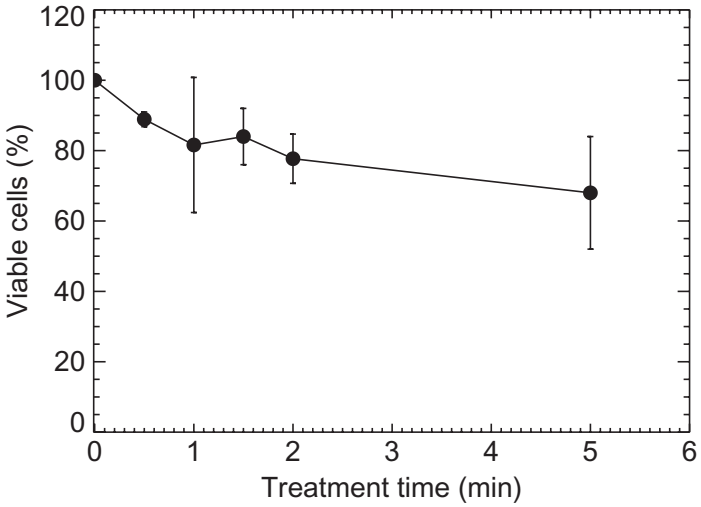

Figure 7. Conjunctival fibroblasts' viability as a function of treatment time (data are expressed as percentage with respect to the control untreated cases).

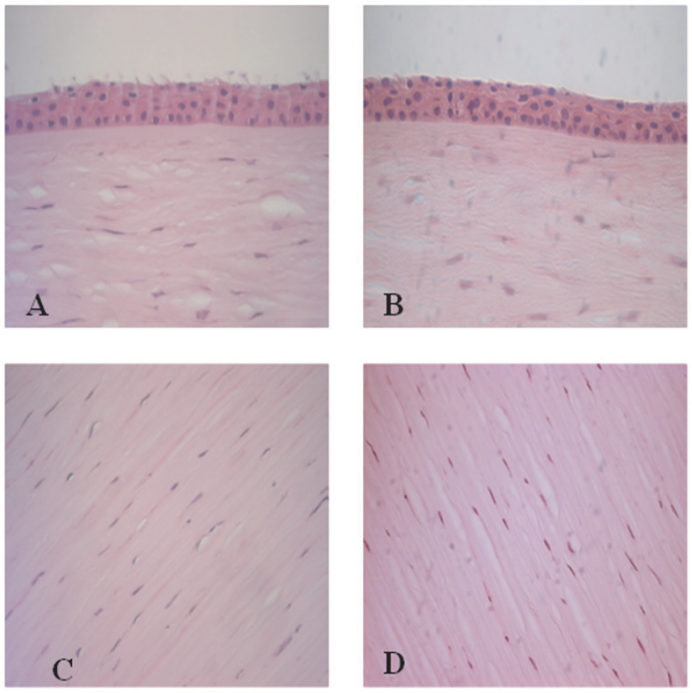

Figure 8. Histological analysis of cornea sample *ex vivo* treated (A, C) or untreated (B, D) with plasma for 2 min. Hematoxylin- and eosin-stained sections were analysed using light microscopy at ×20. A, B: epithelium; C, D: stroma. No morphological changes were observed.

room temperature. The test is based on the fact that only the viable cells, i.e. those containing functioning mitocondria, are able to oxidize MTT, giving a violet-red coloured reaction product. As figure 7 shows, for the tested time intervals cell cultures maintain a viability comparable to that of untreated cells (control cultures).

Finally, the effects of *ex vivo* plasma treatment on human cornea were histologically evaluated. For this purpose, after 5 min, corneas were fixed in formalin, paraffin-embedded, and stained with haematoxylin and eosin. As shown in figure 8 no significant morphological changes were observed when compared with untreated control samples.

In conclusion, we have reported about a new kind of plasma source specifically designed for the treatment of living tissues in biomedical applications. We have demonstrated the capability of the source to kill different kinds of bacteria with decimal reduction times of 1–2 min, while preserving the living cells of the substrate. The likely origin of this selective effect, which is very promising for the potential applications of this technology in clinical practice, is likely to reside in the self-repairing mechanisms of the cells against the action of free radicals.

Possible applications of the source are presently under study. In particular, the possibility of treating corneal infections, which constitute a cause of total loss of sight in the most serious cases, will be the object of future investigations.

References

[1] Fridman G, Friedman G, Gutsol A, Shekhter A B, Vasilets V N and Fridman A 2008 *Plasma Process. Polym.* **5** 503
[2] Laroussi M 2005 *Plasma Process. Polym.* **2** 391
[3] Stoffels E, Flikweert A J, Stoffels W W and Kroesen G M W 2002 *Plasma Sources Sci. Technol.* **11** 383
[4] Stoffels E, Kieft I E, Sladek R E J, van den Bedem L J M, van der Laan E P and Steinbuch M 2006 *Plasma Sources Sci. Technol.* **15** S169
[5] Sladek R E J, Stoffels E, Walraven R, Tielbeek P J A and Koolhoven R A 2004 *IEEE Trans. Plasma Sci.* **32** 1540
[6] Goree J, Liu B, Drake D and Stoffels E 2006 IEEE *Trans. Plasma Sci.* **34** 1317
[7] Laroussi M and Lu X 2005 *Appl. Phys. Lett.* **87** 113902
[8] Hong Y C and Uhm H S 2006 *Appl. Phys. Lett.* **89** 221504
[9] Stoffels E, Aranda Gonzalvo Y, Whitmore T D, Seymour D L and Rees J A 2007 *Plasma Sources Sci. Technol.* **16** 549
[10] Laroussi M and Leipold F 2004 *Int. J. Mass Spectrom.* **233** 81
[11] Arbogast S and Reid M B 2004 *Am. J. Physiol. Regul. Integr. Compd. Physiol.* **287** R698
[12] Sosnin E A, Stoffels E, Erofeev M V, Kieft I E and Kunts S E 2004 *IEEE Trans. Plasma Sci.* **32** 1544
[13] Al-Adhami B H, Nichols R A, Kusel J R, O'Grady J and Smith H V 2007 *Appl. Environ. Microbiol.* **73** 947
[14] Denizot F and Lang R 1986 *J. Immunol. Methods* **89** 271