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DIPARTIMENTO DI FISICA E ASTRONOMIA "GALILEO GALILEI"

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Simulation of dose dependent retardation of colony growth and its impact on cell survival curves based on the damage distributions as predicted by the GLOBLE model.

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Introduction

The field of radiobiology studies how cells and living organisms react when they are irradiated. When cells are exposed to ionizing radiation they can react in many different ways depending on the severity of the damage within the DNA. Many different scenarios can be observed between the two extreme cases in which the cell can continue its normal reproductive cycle or it can be severely injured and lose as a consequence its reproductive integrity. After a given time t the initial cells that were irradiated will produce colonies with different dimensions depending on how much irradiation affected cells and compromised their reproductive cycle. Colony size measurements can then give information on cell survival after irradiation: if a colony exceeds a given number N of cells after a time interval Δt it can be considered as a survivor. Graphic representations of the response of cells to a specific irradiated dose can be useful to understand how much cells are affected. For example survival dose-response curves, that represent the number of surviving cells at a time t after irradiation against the irradiated dose, are usually produced in radiobiological experiments. The fraction of surviving cells depends on the criterion that is used to distinguish survivors from non survivors. The standard criterion used in experimental measurements is to consider as survivors those cells that produce colonies bigger than ~ 50 cells after a time interval of $\Delta t \sim 14$ cells cycle times. This time interval corresponds to around 7 days for rapidly cycling cells such as murine cells characterized by a cell cycle of around 12 hours and to 14 days for slowly dividing cells such as human cells that have a cell cycle time of around 24 hours. Some recently published works as the article by F.Guan et al.[1] performed colony size measurements at a different time after irradiation compared to the standard time interval. The question that raised was what impact could the variation of the time interval Δt to perform colony assays or the variation of the threshold criterion N have on the parameters that characterize the survival curves. Although experimental investigations on this topic are possible they would be very expensive so the first choice was to perform in-silico experiments.
The idea of this thesis is to develop a growth model in order to simulate

colony growth after photon irradiation. In this case some experimental data were available so it was possible to validate the model through a comparison. The idea was to produce survival curves from simulated colony growth data and to derive the parameters that characterize those curves. Then the impact of the variation of Δt and of the threshold criterion N could be analysed sistematically varying the parameters.

In this thesis the modeling of cells growth after irradiation will be developed based on a cell survival model called the Giant LOop Binary LEsion (GLOBLE) model, proposed by Friedrich et al. in 2012.[2] It has been developed with the specific aim to predict cell survival after photon irradiation. The model is based on the assumption that cell survival is directly linked to the spatial distribution of double strand breaks within chromatin structures called giant loops. Two different kinds of lesions can be distinguished: isolated double strand breaks (iDSB) if an individual DSB is induced within a chromatine loop, and clustered double strand breaks (cDSB) if two or more double strand breaks occur in a loop. The parameters defined in the GLOBLE model to describe cell survival will be used in this work to describe the cell growth after photon irradiation.

The thesis is organized as follows:

- Chapter 1 will give a theoretical introduction to radiation physics and biology.

- Chapter 2 will describe radiobiological modeling, particularly focusing on the GLOBLE model. In this chapter experimental results will also be presented.

- Chapter 3 will present the analysis performed to develop and test a cell growth model.

- In Chapter 4 results obtained with simulations will be discussed, and some future perspectives will be proposed.

Introduzione

La radiobiologia studia come le cellule e gli organismi viventi reagiscano all'irradiazione. Quando le cellule sono esposte a radiazioni ionizzanti possono reagire in diversi modi, a seconda della gravità del danno arrecato al DNA. Molti diversi scenari possono essere osservati tra i due casi estremi in cui le cellule continuano a riprodursi normalmente come se non fossero state irradiate, o sono danneggiate tanto da perdere la loro integrità riproduttiva. Ad un tempo t dopo l'irraggiamento le cellule presenti inizialmente, che sono state irradiate, produrranno colonie di dimensioni differenti, a seconda di quanto la radiazione abbia compromesso l'integrità riproduttiva di ogni singola cellula. Misurazioni della dimensione delle colonie danno informazioni sulla sopravvivenza di cellule sottoposte a radiazioni: una colonia viene considerata sopravvissuta se supera un certo numero N di cellule dopo un intervallo di tempo Δt dal momento dell'irradiazione. Rappresentazioni grafiche della risposta delle cellule ad una specifica dose di radiazioni sono utili per capire quanto le cellule siano state danneggiate. Ad esempio le curve dose-risposta, che rappresentano il numero di cellule sopravvissute ad un tempo t dopo l'irradiazione in funzione della dose irradiata, sono solitamente prodotte per descrivere i risultati ottenuti in esperimenti condotti nell'ambito della radiobiologia. La frazione di cellule sopravvissute alle radiazioni dipende dal criterio utilizzato per la selezione. Il criterio standard prevede di considerare come sopravvissute quelle colonie formate da più di 50 cellule dopo un intervallo di tempo pari a $\sim$ 14 cicli di riproduzioe cellulare. Questo intervallo corrisponde a circa 7 giorni per le cellule che si riproducono velocemente, come le cellule murine caratterizzate da un ciclo cellulare di 12 ore, ma può prolungarsi fino a circa 14 giorni per le cellule umane, che hanno un tempo di divisione cellulare di circa 24 ore. In alcuni articoli recentemente pubblicati, come ad esempio quello scritto da F. Gual et al.,[1] le misure delle dimensionidelle colonie sono state effettuate in tempi diversi rispetto a quelli considerati solitamente. A questo punto ci si è chiesti che impatto potesse avere la variazione del tempo a cui prendere le misure, come anche la variazione del criterio con cui distinguere le colonie sopravvis-

sute, sui parametri ottenuti dalle curve di sopravvivenza. Per rispondere a questa domanda si sarebbero potuti svolgere alcuni esperimenti, che però sarebbero risultati molto costosi. Si è dunque scelto di procedere per una prima analisi con degli esperimenti in-silico. L'idea su cui si basa questa tesi è di sviluppare un modello di crescita cellulare per simulare la crescita di colonie cellulari dopo l'irradiazione con fotoni. In questo caso alcuni dati sperimentali sono stati resi disponibili, dunque è stato possibile validare il modello tramite un paragone tra simulazioni e dati sperimentali. L'idea di partenza era cercare di riprodurre le curve di sopravvivenza a partire dai dati di crescita delle colonie ottenuti con simulazioni, e di derivare poi i parametri che caratterizzano queste curve. Successivamente è stato studiato l'impatto della variazione di N e Δt variando sistematicamente questi due parametri. In questa tesi il modello di crescita cellulare dopo l'irradiazione verrà sviluppato sulla base di un modello di sopravvivena cellulare chiamato GIant LOop Binary LEsion (GLOBLE), proposto da Friedrich et al.[2] nel 2012. Il modello GLOBLE è stato sviluppato con lo scopo specifico di riprodurre i dati di sopravvivenza cellulare dopo l'irradiazione con fotoni. Esso si basa sull'assunzione che la sopravvivenza delle cellule sia direttamente legata alla distribuzione spaziale delle doppie rotture della catena del DNA, chiamate DSB o double strand breaks, all'interno di strutture fatte di cromatina dette giant loops. Possono essere distinti due tipi di lesioni a livello del DNA: le lesioni vengono definite isolate (iDSB) se una sola DSB avviene all'interno di un loop di cromatina, vengono invece definite complesse (cDSB) se due o più DSB avvengono in un loop. I parametri definiti nel modello GLOBLE per descrivere la sopravvivenza delle cellule alla radiazione fotonica saranno usati in questo lavoro di tesi per descrivere la crescita delle colonie.
La tesi si articola come segue:

- Nel Capitolo 1 si trova un'introduzione teorica ai concetti di fisica e biologia utili nell'ambito della radiobiologia.

- Nel Capitolo 2 sarà descritto in particolare un modello rediobiologico chiamato GLOBLE. Alcuni risultati sperimentali saranno inoltre esposti in questa sezione.

- Nel Capitolo 3 sarà presentata l'analisi fatta per sviluppare e testare un modello di crecita per le cellule.

- Nel Capitolo 4 saranno discussi i risultati ottenuti per mezzo delle simulazioni, e saranno proposte alcune prospettive future.

Chapter 1

Radiation physics and biology

1.1 Interaction of radiation with matter

Radiobiology is a field of science that studies the effect of ionizing radiations on biological tissues and living organisms. To perform radiobiological experiments, given a certain type of radiation, it is very important to quantify how much energy is transferred to the traversed matter.

The physical quantity that gives this information is the absorbed dose, defined as the mean energy imparted by ionizing radiation to matter per unit mass. The unity of measurement is called Gray (Gy) where 1Gy= 1 J/kg.

Radiation is said to be ionizing if it is energetic enough to ionize atoms and molecules of the target. Ionizing radiation can be classified as electromagnetic or particulate.

Electromagnetic radiation, such as x-rays or γ-rays, interact with matter through three main processes:[3]

- Photoelectric effect: a photon interacts with a bound electron of an atom of the traversed material and transfers all its energy to the electron that is then emitted.

- Compton effect: the photon interacts with an outer orbital electron and transfers part of its energy to the electron. The photon is then scattered with a lower energy.

- Pair production: if the photon's energy exceeds 1.022 MeV it can be converted to the mass and kinetic energy of an electron-positron pair. The positron subsequently annihilates and two annihilation photons are produced.

The electrons emitted in the different processes collide with other electrons within the target causing ionization or excitation of atoms.

Charged particles interact with the electrons in the target through electromagnetic interaction. These electrons can be excited or ionized depending on the ion's energy.

Photons are most commonly used in clinics to treat patients but also protons or heavy charged particles, such as carbon ions, are used in some specialized facilities. To be effective in radiotherapy the heavier particles must be accelerated to energies of hundreds of MeV.

Ionizing radiation can cause a biological damage to cells. The severity of this damage depends on the local rate of energy deposition along the particle track, that is called Linear Energy Transfer (LET), defined as $L = dE/dl$, where dE is the average energy locally imparted to the medium by a particle of a given energy traversing a distance dl. In radiobiology the LET is usually measured in $keV/\mu m$.

1.2   DNA structure

There is strong evidence that the biological effects of radiation are mainly due to DNA damage. The DNA is a long molecule made of smaller structural units called nucleotides. It stores all the genetic information that is necessary for protein biosynthesis. As illustrated in Fig.1.1 the DNA is composed by two polynucleotide strands linked by hydrogen bonds between the complementary bases. The strands are coiled around each other to form a double helix structure with a diameter of 20Å and a pitch of 34Å. This coiled structure is further packed and ordered by proteines called histones into structures called nucleosomes, that consist of about 150 base pairs (bps). Histones are the main protein components of chromatin that is a complex of macromolecules consisting of DNA, proteins and RNA. The structure of the condensed chromatin is arranged into independent loops that are then organized into structures called chromosomes. The human genome is composed by 23 chromosomes and the biggest one contains approximately 220 Mbp. Many studies have investigated the importance of chromatin's structure with respect to the repairability of DNA double strand breaks. For example, some research groups proposed to define as Giant Loops those chromatin loops that are formed by several megabase pairs, and suggested that these structures could be relevant for cell response to radiation damage.

When cells are exposed to radiation the DNA can be damaged either directly or indirectly.

Direct damage happens when the radiation interacts directly with the critical target through ionization, excitation or Coulomb interaction with the atoms of the DNA.
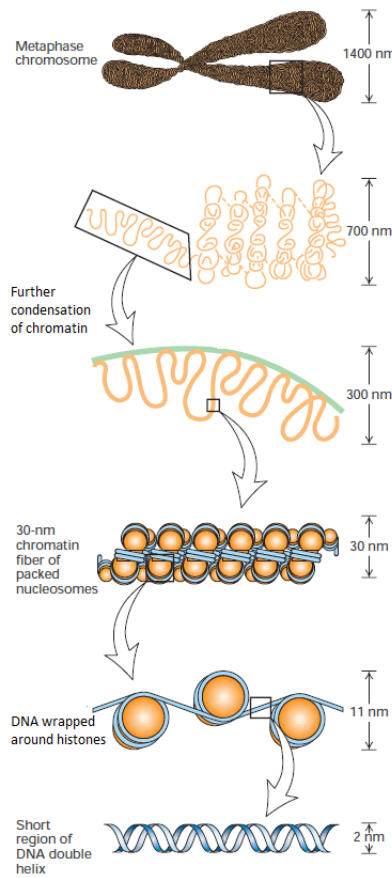
Figure 1.1: Organization of Euchariotic chromosomes, adapted from "Molecular Cell Biology"[4] .
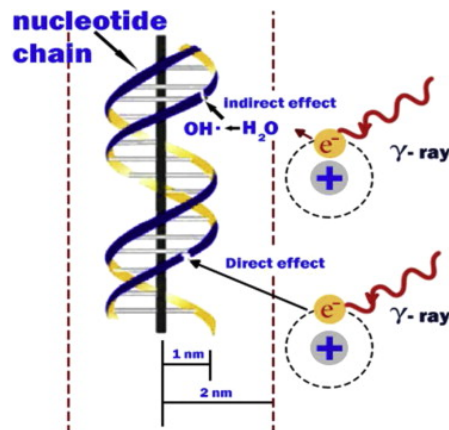

Figure 1.2: Illustration of direct and indirect damage to the DNA, adapted from "Radiobiology for the Radiobiologist".[5]

Indirect damage happens when the radiation interacts with other components of the cell and as a result free radicals are produced. These ones can diffuse in the cell and damage the DNA in later interactions. We can take as an example some X-rays interacting with a component of the cell. Through photoelectric effect, Compton effect or pair production a high energy electron is emitted and, interacting with water molecules, it causes the production of free radicals. These radicals can break some chemical bonds of the DNA and damage the DNA structure.

A break of a chemical bond in a single strand, called single strand break (SSB), is easily repaired using the opposite strand as a template. It may result in a cell mutation if the repair process is incorrect but usually SSBs can be neglected as far as cell killing is concerned. If breaks occur in both strands and they are directly opposite or separated by a few base pairs, a double strand break (DSB) takes place. This kind of lesion is more difficult to repair and it can result in cell killing, cell mutation or carcinogenesis. The yield of double strand breaks in irradiated cells is about 0.04 times the yield of single strand breaks.

1.3   Cell cycle

The life of each cell is characterized by a cycle of events that lead to cell proliferation. For mammalian cells the cycle is composed by two main processes:

- Mitosis (M)

- DNA synthesis (S)

Mitosis is the process of cell division when replicated chromosomes are separated into two new nuclei that carry the same genomic information.

Mitosis and synthesis are separated by two time gaps called G1 and G2. In the first gap the cell duplicates its cellular content (excluding chromosomes) and in the second gap the cell repair mechanisms can fix possible errors in the duplicated chromosomes.

The cell cycle time T_{cell} varies between different mammalian cells going from about 11 hours for Chinese hamster cells to hundreds of hours for stem cells.

Within a culture cells are said to be synchronous if they are in the same phase of the cell cycle at the same time, on the other hand asynchronous cultures are composed by cells that are at different stages of their cell cycle.

When cells are exposed to ionizing radiation they can be damaged. The first effects are the physical and chemical ones that take place after 10^{-16} to
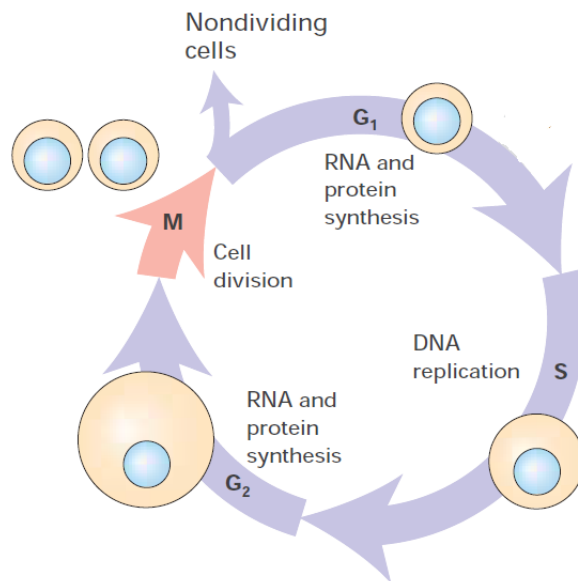
Figure 1.3: Illustration of cell cycle for mammalian cells, adapted from "Molecular Cell Biology"[4] .

10^{-1}s . The biological effects appear from seconds to hours after irradiation. Most of the lethal events are due to damages to the DNA, which is the critical target in the cell. The eventual clinical effects due to the DNA damage can be observed even after years.

After cells irradiation many different scenarios may occur:

- The cell continues its normal reproductive cycle, forming large colonies. This cell is said to be clonogenic.

- The cell is able to proliferate, but a time delay in its cell cycle is observed.

- The cell goes through a few divisions forming small colonies and then it is not able to proliferate any longer. This cell is considered as dead.

- The cell is not able to divide anymore and it dies.

Cell survival after irradiation is measured by clonogenic assays. A primary cell culture is irradiated and some measurements of colony size are usually taken after an incubation time from one to two weeks. The number of cells that form the colony can be directly counted using a microscope or it can be derived measuring the area occupied by the colony. Assuming that colonies
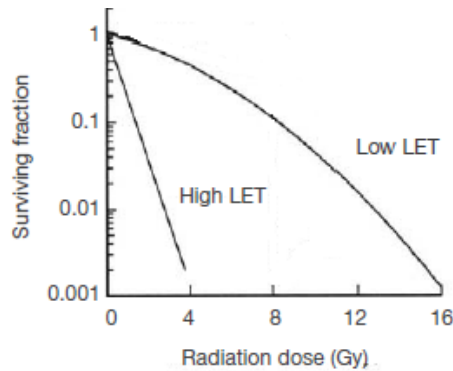
Figure 1.4: Example of survival curve for high LET and low LET radiation, adapted from "Radiobiology for the radiobiologist"[5] .

grow in a monolayer, the total area occupied by cells can be divided by the average size of a cell yielding the number of cells in the colony.

The loss of the cell's ability to proliferate as a function of radiation dose is described by dose-survival curves.

An example of dose-survival curve is presented in Fig. 1.4. The type of radiation influences the final shape of survival curves: for low LET radiation the curves show an initial slope followed by a shoulder region and then become nearly straight at higher doses.

To compare the relative biological effectiveness of different kinds of ionizing radiation a quantity called RBE can be introduced. Given the amount of dose D_R for radiation of type R, necessary to cause a certain amount of damage to cells or tissue, the RBE is defined as the ratio $RBE = D_X/D_R$ where D_X is the dose of radiation of type X necessary to cause the same biological effect. Since many experiments have been performed with photons D_X is usually the dose for photon radiation. For instance, in Fig. 1.4, RBE is ~ 4 at lowest survival level.

Through the years many biophysical models have been proposed to describe the shape of survival curves of mammalian cells. Among those the Linear Quadratic (LQ) model considers two different components for cell killing after irradiation. The first one is proportional to the dose and the second one is proportional to the square of the dose. As a result the fraction of surviving cells S after an irradiation with a dose D is expressed by the formula

$$S = e^{-\alpha D - \beta D^2} \tag{1.1}$$

where α describes the initial slope of the cell survival curve and β describes the quadratic component of cell killing. This model however has some lim-

itations in the high dose region. A new model called GLOBLE[2] has been developed to overcome this problem in the description of the slope of survival curves in the high-doses region.[2] The GLOBLE model only describes the case where photon radiation is used.

Another model, called LEM (Local Effect Model)[6–8] , can be used to reproduce survival curves for ion irradiation. This model is based on the assumption that lethal damages to the DNA cause cell death, and every lethal event is considered as a consequence of local damage to the DNA. The calculation of the biological effect of a certain radiation is based the local amount of energy deposited by primary particles in the cell nucleus, considering that equal deposited local doses should lead to equal local effects independent on the radiation used. Knowledge of the photon dose response curve is then sufficient to predict the effects of ion irradiation.

Chapter 2

Materials and Methods

2.1 Radiobiological modeling

2.1.1 GLOBLE model

The Giant LOop Binary LEsion (GLOBLE)[2] model is used to describe cell survival after photon irradiation. One of the main assumptions of the model is to consider that within the cell nucleus chromatin is organized in megabase pair-sized loops also called "Giant Loops". We can assume in a first approximation that the DNA is homogeneously distributed in the cell nucleus and the amount of about 2 Mbp corresponds to an average cubical subvolume with approximately 0.5 μm side length. The volume of the cell nucleus is around 500 μm^3.

Double strand breaks are supposed to be the main source of damage for the cells. The GLOBLE model distinguishes between two different situations represented in Fig. 2.1 :

- isolated DSB (iDSB), when an idividual DSB is induced within a loop.

- clustered DSB (cDSB), when two or more DSBs are induced within a loop.

In the first case it is easier to repair the lesion because DNA ends are close together and both sides of the DSB are still attached to the nuclear matrix. In the second case DNA fragments are produced due to multiple double strand breaks and they easily diffuse away from their original position. In this situation the repair mechanisms might not be able to repair the lesion because of the big distance between the loop segments. As a consequence clustered DSBs represent a much more severe damage to the cell. Lesions induced in different chromatin loops are assumed to be processed
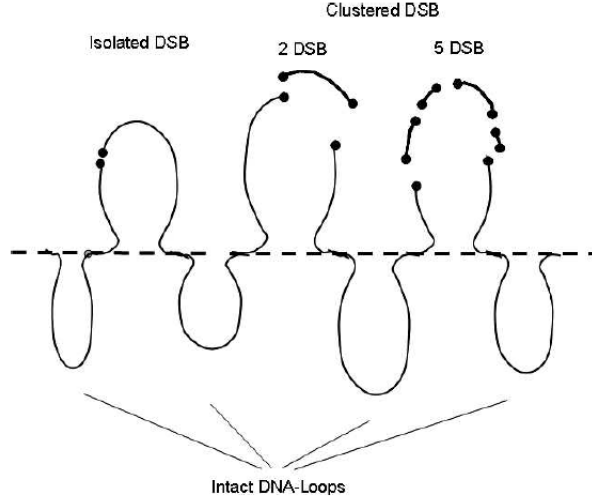
Figure 2.1: Representation of isolated and clustered double strand breaks within chromatin loop in the DNA taken from the article of T.Fiedrich et al.[2]

independently. Different lethalities can be attributed to iDSBs and cDSBs: ε_i and ε_c represent respectively the average number of lethal events of an isolated DSB or a clustered DSB. According to the higher severity of clustered DSBs ε_c is expected to be much higher than ε_i .

Since both ε_i and ε_c are smaller than 1 they can be considered as a measure of the probability for an iDSB or a cDSB to be lethal.

The survival probability S can be found using the formula

$$S = e^{-(n_i\varepsilon_i + n_c\varepsilon_c)} \tag{2.1}$$

where n_i and n_c are respectively the average number of chromatin loops with isolated or clustered DSBs.

According to the experimental results obtained using hard x-rays and γ-rays, the initial yield of DSB is set to $\alpha_{DSB} = 30$ DSB per Gy and per cell. The yield is independent of the cell type. Defining S_G as the size of the whole genome and S_L as the size of a single chromatin loop, the total number of loops contained in the genome is $N_L = S_G/S_L$ and the frequency of induction of DSB is $Y_{Loop} = \alpha_{DSB}/N_L$ per Gy and per loop. All further results were obtained considering $S_G = 6 \cdot 10^9$ bp and $S_L = 2$ Mbp. The resulting number of loops is $N_L = S_G/S_L = 3000$ per cell. Using Poisson statistic we can finally determine

$$n_0 = N_L \cdot e^{-Y_{Loop}D} \tag{2.2}$$

$$n_i = N_L(Y_{Loop}D) \cdot e^{-Y_{Loop}D} \qquad (2.3)$$

$$n_c = N_L - n_0 - n_i \qquad (2.4)$$

where D is the dose and n_0, n_i and n_c represent respectively the number of loops containing exactly zero DSB, exactly one DSB or more than one DSB.

These numbers can be substituted in formula 2.1 to find the dose-response curves.

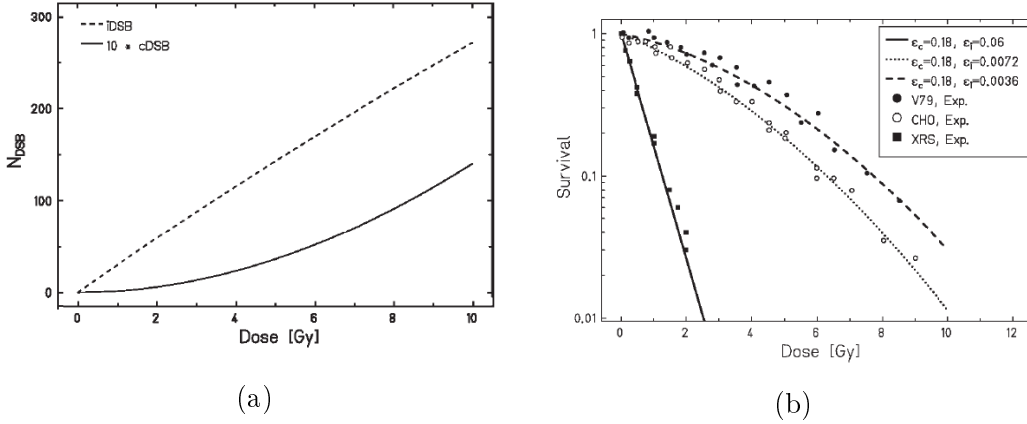|	
(a)	(b)

Figure 2.2: In (a) the number of isolated and double strand breaks derived with the GLOBLE model is shown as a function of the irradiated dose. Fig (b) presents an example of survival curves produced by the GLOBLE model with different ϵ parameters, fitted to experimental data belonging to three different cell lines called V79,CHO and XRS. Both graphs are taken from the article from Friedrich at al.[2]

In Fig. 2.2 (a) the number of isolated and clustered DSBs derived with the GLOBLE model is illustrated as a function of the dose. Since clustered lesions are more rare than isolated ones, the number n_c has been multiplied by a factor of ten to allow a significant comparison between the two curves.

Fig. 2.2 (b) taken from the article[2] by Friedrich et al. shows how the GLOBLE model fits experimental data obtained using different cell lines and the values of ε_i and ε_c.

2.2 Experimental data

Through all this work simulations of colony growth after irradiation with photons have been qualitatively compared to some experimental data re-

ported by G.Böhrnsen.[9] He measured cell growth after irradiation using x-rays obtained with a linear accelerator operated at 6 MV. The absorbed dose considered for the experiment varies in a range from 0 to 5 Gy. For his measurements he used in vitro V79-4 Chinese hamster lung cells, that are characterized by a high plating efficiency and by a cell cycle time of around 12 h. Referring to the GLOBLE model parameters, cell survival of V79 hamster cells is described by $\epsilon_i = 0.0036$ and $\epsilon_c = 0.18$.[2] Colony sizes were measured 1, 2, 3 and 5 days after irradiation.

Due to large dimensions it is not feasible to count cells individually with a microscope, so for big colonies the total number of cells is usually derived from the total area occupied by a given colony. The scaling factors to calculate the cell number from the occupied area were determined from manual counting of cell numbers for colonies up to 200 cells/colony. Survival was then calculated from the fraction of cells developing colonies bigger then 50 cells per colony. At every cell cycle each cell splits into two cells so every distribution is expected to be peaked around the value $2^{t/T_{cell}}$, where t is the time lapse between irradiation and measurement and T_{cell} is the cell cycle time. This is exactly what can be observed in Fig. 2.3 at Day 1 for every absorbed dose. It is noticeable that experimental data show broader distributions than the ones that we obtain simply considering an exponential growth model. This is due to the fact that cells are asynchronous, so they can split at earlier or later times than exactly T_{cell} hours after irradiation. This means that colonies can be a little bit bigger than $2^{t/T_{cell}}$.

Another factor that has to be considered is the uncertainty in the value of colony size due to the method of measurement. Since the number of cells in the colony is deduced from the occupied area it is possible that the estimated number of cells is slightly different from the actual number of cells in the colony.

Due to irradiation some colonies show a delay in cell growth, which means that at a certain time colonies are smaller than expected for control cells. This effect is visible at higher doses (3 Gy or 5 Gy) because in this case there is a higher number of double strand breaks so cells are more affected. In the experimental data, for example, at Day 5 for 3Gy and 5Gy, the peaks of the distributions are shifted to 512 cells and 256 cells respectively, compared to the expected value for control cells of 1024. As shown in Fig. 2.4 at Day 5, for an absorbed dose of 2 Gy 3 Gy or 5 Gy, two different distributions can be distinguished. The one that is situated on the left hand side of the graphs represents all those cells that have a strong time delay in cell cycle time and as a result they can only form small colonies. The other cells that have a small time delay or are completely unaffected in their reproductive cycle can produce bigger colonies and they contribute to the distribution on

the right hand side of the graphs. Experimental colony growth curves were also reported by G.Böhrnsen.

In Fig. 2.5 growth curves are presented for control cells in (a) and for an irradiate dose of 5 Gy in (b). Within each graph a single curve represents how an initial cell develops in time forming a colony through its reproductive cycle. Control cells, represented in (a) grow exponentially. Cells irradiated with 5 Gy show different behaviours: some colonies grow exponentially, whereas some others show a delayed growth and eventually tend to a constant size after 100 hours. A few curves in this case go to zero, meaning that colonies were not measurable experimentally.
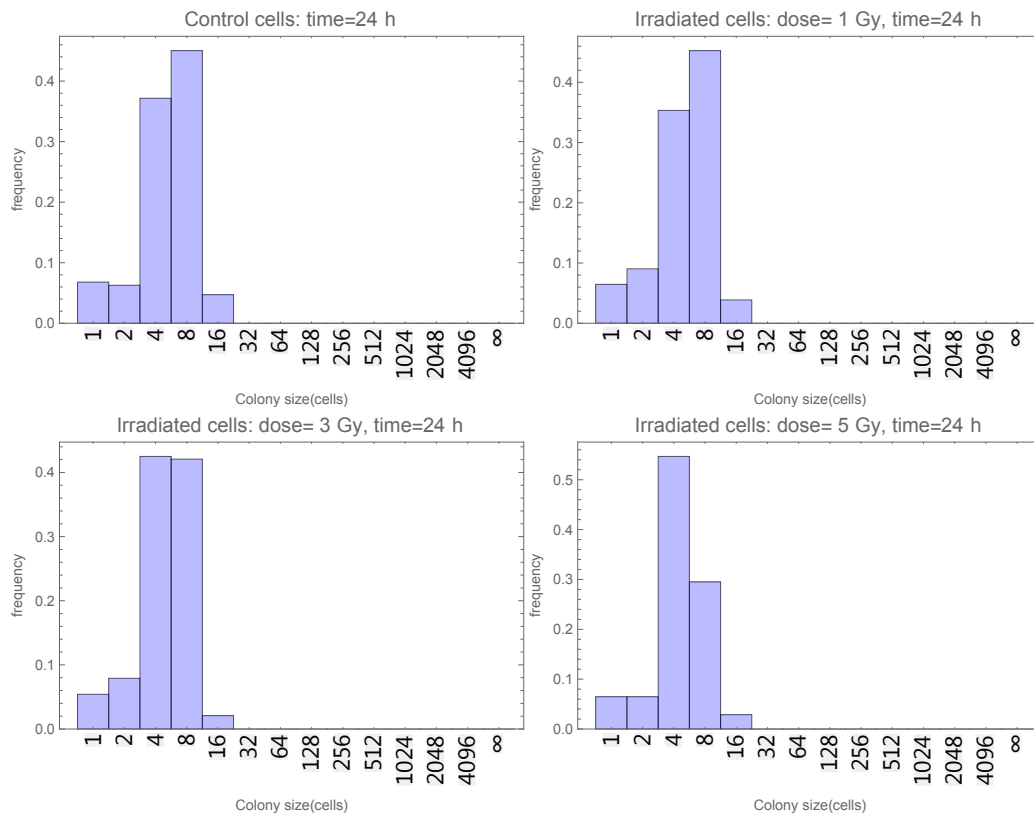

Figure 2.3: Representation of colony size distribution measured experimentally 24 hours after irradiation. Different graphs correspond do different irradiated doses in a range from 0 to 5 Gy.
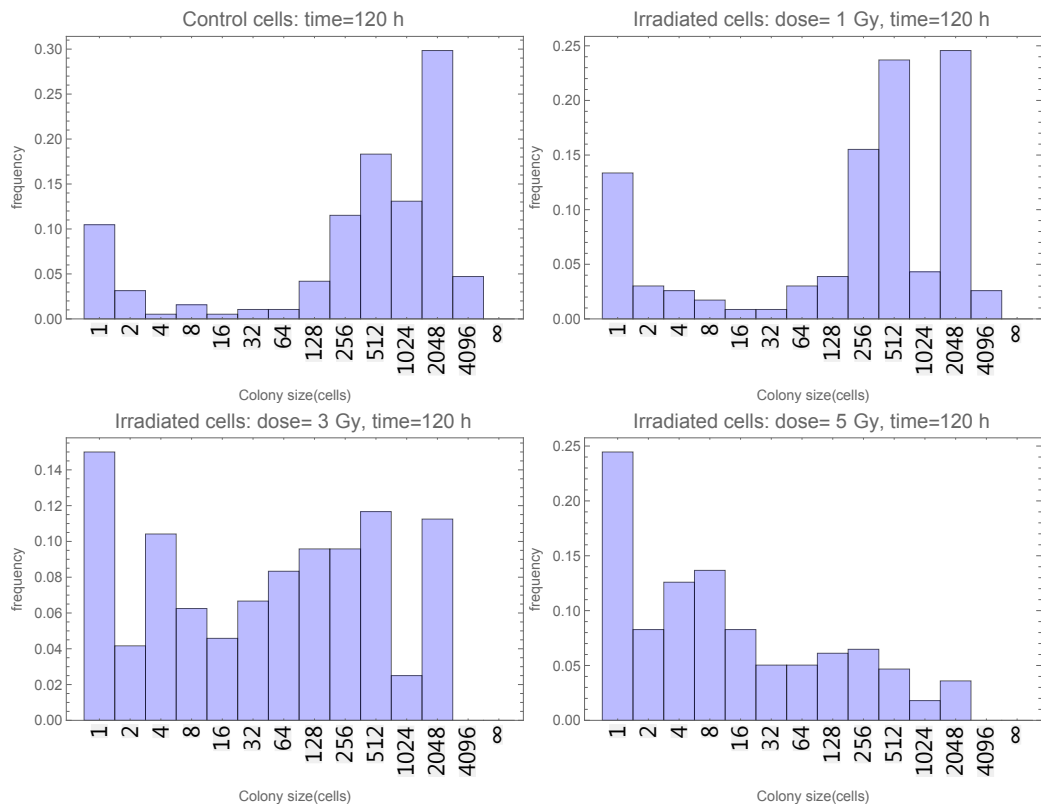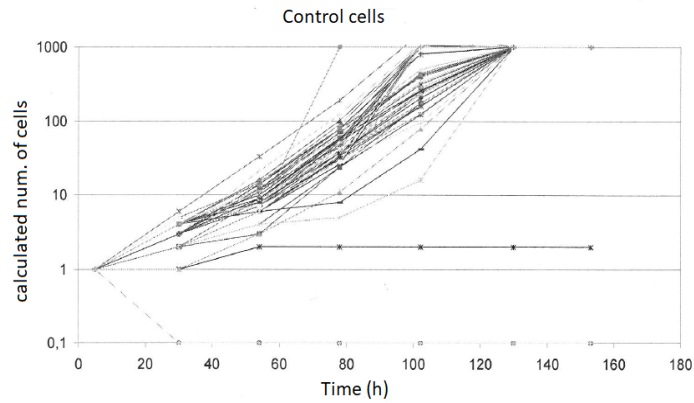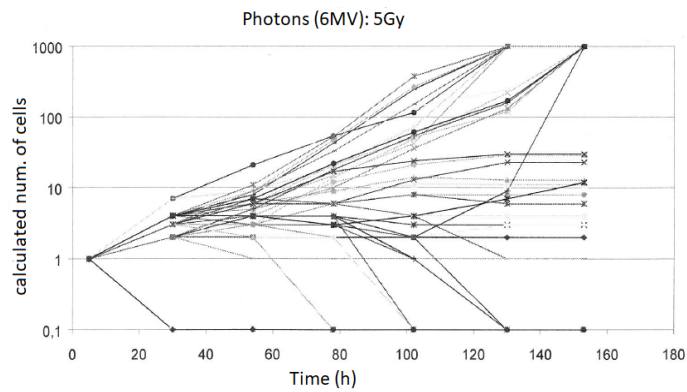
Figure 2.4: Representation of colony size distribution measured experimentally 120 hours after irradiation. Different graphs correspond do different irradiated doses in a range from 0 to 5 Gy.

(a)

(b)

Figure 2.5: These graphs, taken from the work of G. Börnsen, show colony growth at increasing times respectively for control cells on the left and for an irradiated dose of 5 Gy on the right. Each curve represents an individual cell.

Chapter 3

Modeling cell growth

In this work of thesis many different aspects regarding colony growth after irradiation have been investigated. The first step of the analysis was to find a model to describe cells growth and the time delay that is observed in colony growth due to irradiation. The second step was then to simulate with the proposed model survival curves and to compare the results to the linear quadratic model, that will from now on will be referred to as LQ model. Another important feature was to study the impact of different threshold criteria to define surviving colonies on survival curves. The final step was to produce cell growth curves and to compare them to the ones reported by G. Böhrnsen.

At the beginning of the development of a growth model it is important to know the general law that describes how colonies are expected to grow in time. The simplest equation that gives the number of cells at a time t after irradiation is

$$N_{cells}(t) = 2^{t/T_{cell}} \tag{3.1}$$

where T_{cell} is the cell cycle time. From the experimental data we can see that some colonies, especially at higher doses, show a delay in colony growth due to irradiation. To reproduce that behaviour with a growth model we suggest to add a time delay T_{del} to cell cycle time. The new equation describing the total number of cells after a time t can then be written in the form

$$N_{cells} = 2^{t/(T_{cell}+T_{del})} \tag{3.2}$$

The time delay should be proportional to the total amount of damage to the DNA so that a more severe damage leads to a stronger time delay. The total effect is used to describe time delay because it is characterized by a

non-linear dependency on the dose, which reflects the fact that time delay does not depend linearly on the dose.

The time delay is expected to be zero for unaffected cells that can continue their normal reproductive cycle and to go to infinite for high doses where cells are severely injured and are not able to divide anymore.

This time delay should be described by a proper combination of the same parameters used by the GLOBLE model, that are basically the number of isolated and clustered double strand breaks n_i and n_c and the lethalities ϵ_i and ϵ_c.

In order to point out the main requirements that the model had to fulfill, the method that was used through all the analysis will be briefly described. At first it was necessary to write an equation for time delay, which means that the parameters that determine time delay had to be identified among the parameters used by the GLOBLE model. Different parameters have been combined to obtain an equation for time delay and then colony size distributions were simulated varying the irradiated dose. Then the model has been refined in order to obtain closer results to the experimental data. At last survival curves have been produced and the α and β values were derived to have a direct comparison with the Linear Quadratic model.

3.1   First models to describe colony growth

3.1.1   First Growth model: time delay dependency on the total effect.

The very first attempt was to define the time delay as

$$T_{del} = c\,\epsilon \tag{3.3}$$

where c is a constant value and ϵ is the total effect. The total effect is given by the formula

$$\epsilon = n_i\,\epsilon_i + n_c\,\epsilon_c \tag{3.4}$$

where ϵ_i and ϵ_c are the lethalities and n_i and n_c the number of loops affected respectively by one DSB or two or more DSBS. The total effect describes the total amount of damage due to both isolated and clustered DBSs.

An initial set of a hundred cells is usually taken to perform survival experiments. The idea was to assign to each initial cell a value of ϵ so that every cell would in the end be characterized by a certain time delay. Since both n_i and n_c follow a Poissonian distribution, two sets of a hundred values

randomly drawn from two Poissonian distributions were produced taking as mean values n_i and n_c given from Eq. 2.3 and 2.4. A hundred ϵ values were then obtained adding the nth element of the first term with the corresponding n^{th} element of the second term of the sum. Replacing these ϵ values in Eq. 3.3, a value of time delay for every initial cell could finally be provided.

In Fig. 3.1 two histograms showing the time delay distribution for different irradiated doses are compared. The graph on the left is relative to an irradiated dose of 1 Gy, whereas a dose 5 Gy has been used to produce the graph of the right. For this simulation the constant c was set to 2 in order to have a time delay of around 0.5 h per Gy, according to the results in the article of G.Böhrnsen's.[9] The model used for this first analysis, characterized by a time delay $T_{del} = 2\,\epsilon$, will be referred to as GM1 (Growth model 1).
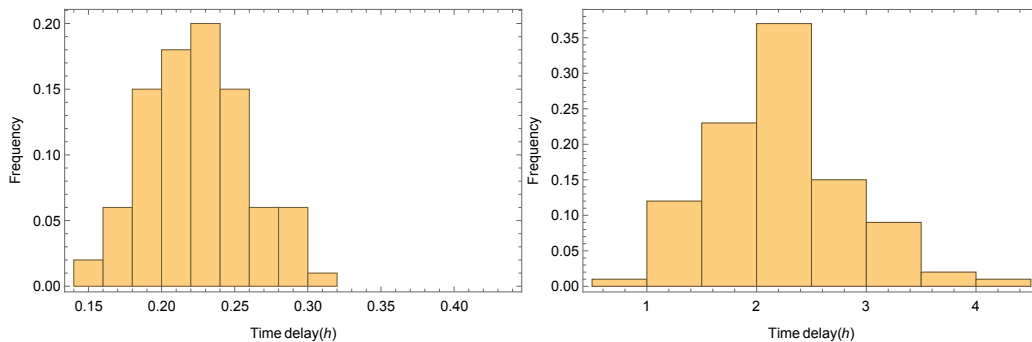

Figure 3.1: Analysis of time delay distribution using GM1. As an example the time delay distribution are here illustrated using an irradiated dose of 1 Gy for the graph on the left and of 5 Gy for the graph on the right.

In Fig. 3.1 it is clearly visible that time delay does not depend linearly on the dose. In fact, considering a dose of 5 Gy, the time delay distribution is peaked at a value which is around ten times higher than the value obtained with an irradiated dose of 1 Gy. This is due to the fact that the damage caused by clustered double strand breaks, that is included in the definition of time delay, has a quadratic dependence on dose as it can be seen in Fig. 2.2. With this model then colony growth histograms were simulated to have a direct comparison with experimental data. As presented in Fig. 3.2 the distributions' shape is not similar to the experimental results, especially looking at 120 h after irradiation. Furthermore even if the peaks' position are well reproduced by this model, colony size distributions are much more spread in the real data compared to simulations. This can be explained looking at the stochastic distribution of lethal events. The narrow distribution of colony
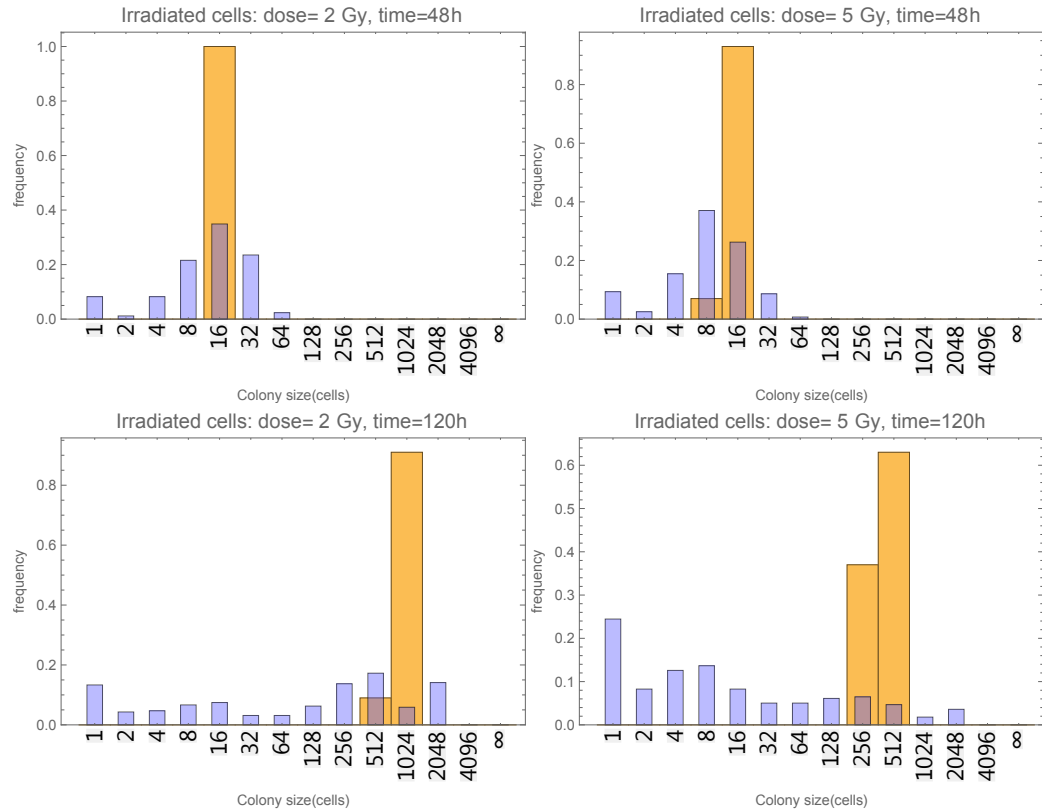
Figure 3.2: These histograms represent colony size for irradiated doses of 2 Gy and 5 Gy at different times after irradiation. The experimental distributions are visualized in blue and the simulated ones in orange.

size in simulated data reflects the narrow range of ϵ values produced using this model. To obtain more spread distributions it is necessary to have a wider range of time delays that means a wider range of ϵ values.

3.1.2   Comparison with experimental data

In this section a brief description on how experimental data have been compared to simulations is given. Since it was not possible to directly fit simulations with experimental data for the analysis, the comparison has been performed by visual inspection. Simulated and experimental plots of colony size distribution and of colony growth curves were visually compared in order to assess if the overall picture presented the same features. This means that, for example, colony sizes were simulated for different values of c using GM1 and other time delay models in a later stage. At first an approximate value

for c was obtained simulating colony size distribution starting from c=0 and then increasing c in steps of 5. The gross c value found with this procedure was refined increasing and decreasing c in steps of 0.5. At this stage of the analysis it did not make sense to finely tune the parameters with a higher resolution because changes of less than 0.5 for c lead to very similar colony size distributions.

3.1.3 Second Growth model: time delay dependency on the effect due to cDSBs.

Since the width of colony size distributions did not resemble real data, the analysis proceeded with the simulation of the extreme scenario in which time delay would only depend on the damage due to clustered DSBs. In this case the delay was expresses by the formula

$$T_{del} = c\, n_c \epsilon_c \tag{3.5}$$

After checking different values for c the constant was set to a value c=9, that allowed to reproduce well the position of the peaks. The model described by $T_{del} = 9\, n_c \epsilon_c$ will be from now on be called GM2. As shown in Fig. 3.4 colony size distributions are more spread compared to the previous model. The larger width of those distributions can be explained by the fact that the number of clustered DSB is smaller that the number of isolated DSB that was included in GM1. Considering only rare events leads to Poissonian distributions characterized by a larger relative spread. Looking at the results obtained at Day 5 the shapes are not similar to what can be observed in experimental data. It is noticeable in fact that small colonies that are present at Day 5 in experimental data are not reproduced by this model.

Considering all these results it can be concluded that the parameters that have been used used so far did not give a suitable description of time delay.
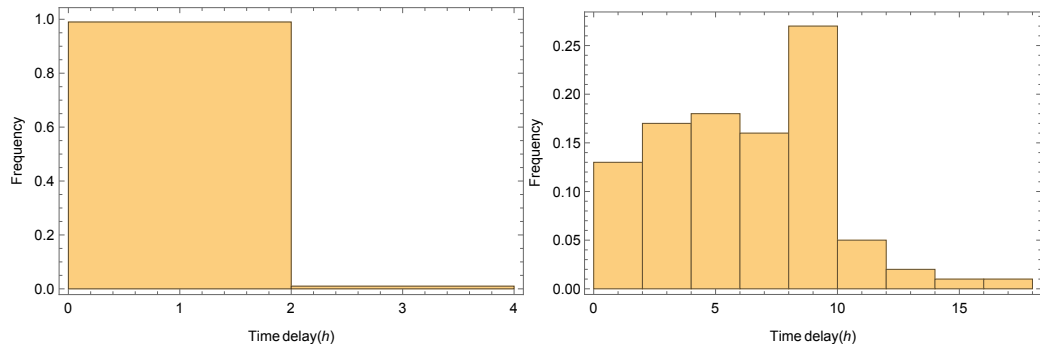
Figure 3.3: Two time delay distributions obtained with model GM2 are presented. The graph on the left refers to an irradiated dose of 1 Gy whereas the graph on the right refers to an irradiated dose of 5 Gy.
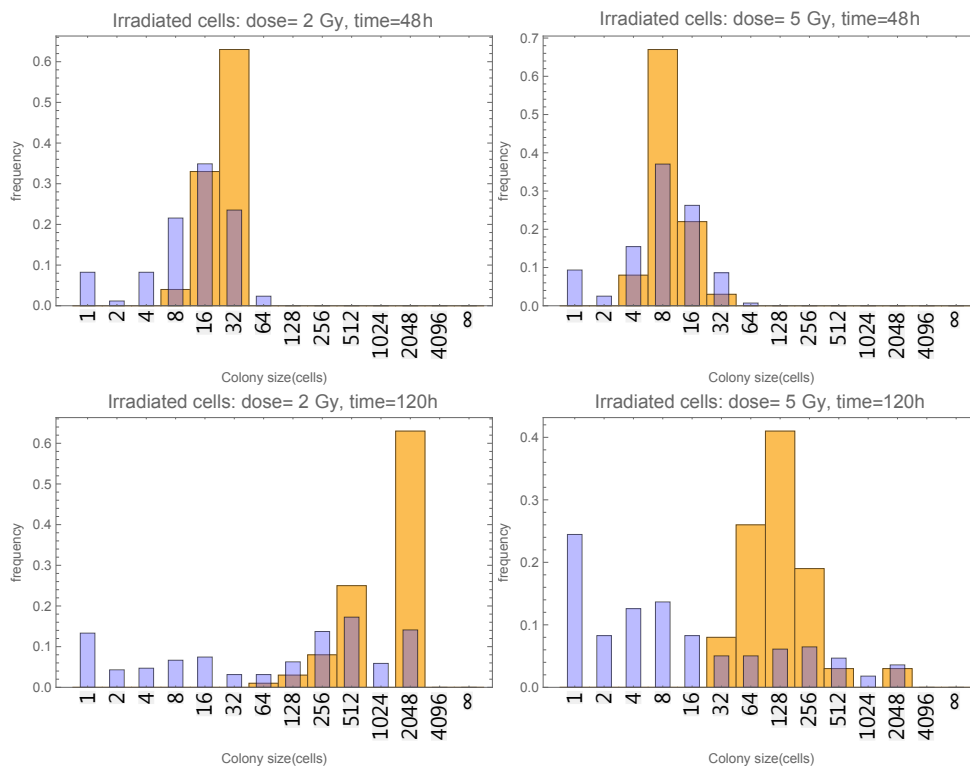

Figure 3.4: These histograms obtained with GM2 represent colony size for irradiated doses of 2 Gy and 5 Gy at different times after irradiation. The experimental distributions are visualized in blue and the simulated ones in orange.

3.2 Third model: a new method to derive the total effect

The main idea that led to the development of a better model was to obtain the total effect ϵ using a different approach. This time the total effect was composed by a set of 100 values randomly drown from a Poissonian distribution with mean $\mu = n_i\epsilon_i + n_c\epsilon_c$. In this case n_i and n_c were not distributions but single values directly taken from equations 2.3 and 2.4. From now on the total effect derived with this method will be referred to as ϵ_{tot}. The new equation for time delay is

$$T_{del} = c\,\epsilon_{tot} \tag{3.6}$$

and the corresponding distributions are presented in Fig. 3.5.
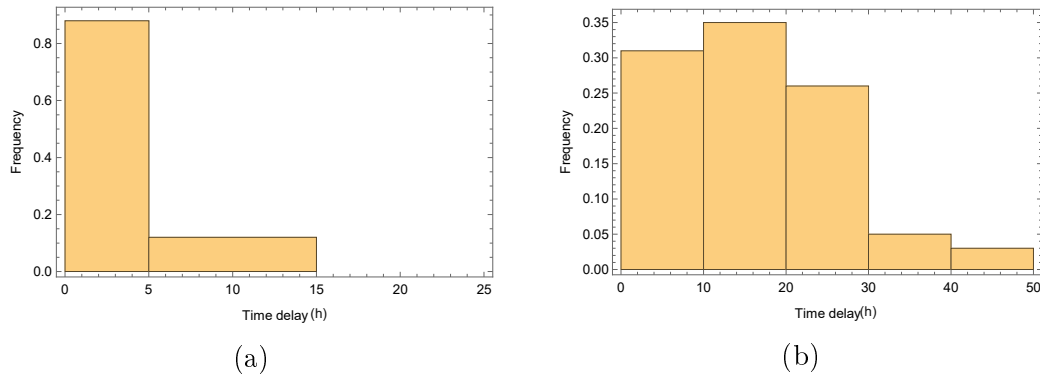
(a)

(b)

Figure 3.5: Comparison between time delay distribution obtained with $T_{del} = 10\,\epsilon_{tot}$ for an irradiated dose of 1 Gy in (a) and 5 Gy in (b).

These new time delay distributions are much broader than those obtained with the previous model. This is due to the fact that the total effect can assume values in a wider range.

With the new expression for time delay, histograms were produced showing frequency versus colony size at different doses and different times after irradiation. Simulations were done with different values for the constant c and c=10 turned out to be the most suitable one. The model characterized by $T_{del} = 10\,\epsilon_{tot}$ will be called GM3. In Fig. 3.6 some representative histograms are shown.
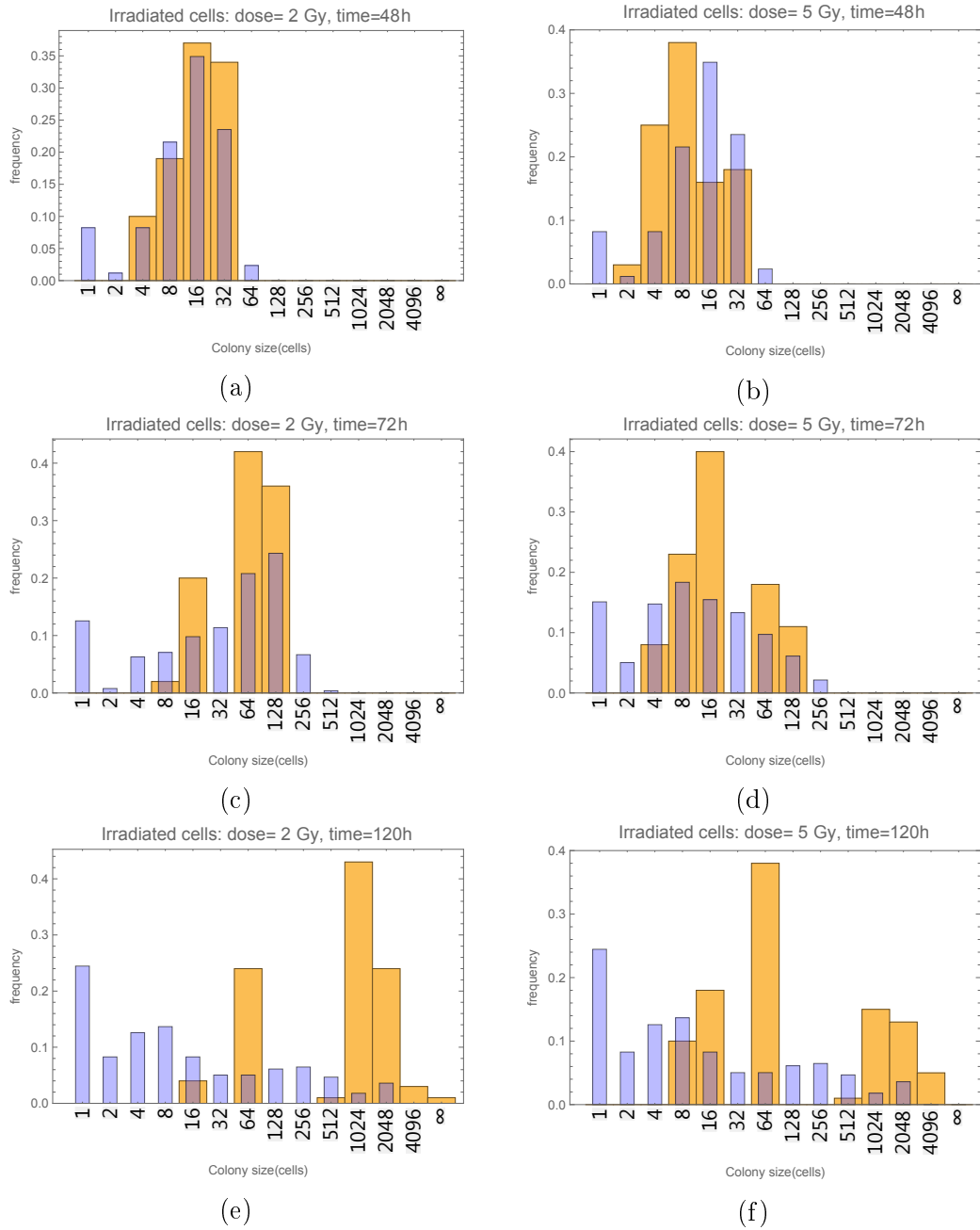
(a)

(b)

(c)

(d)

(e)

(f)

Figure 3.6: Simulation of colony sizes 2, 3 and 5 days after irradiation using GM3, considering two different irradiated doses. Panels (a), (c) and (e) represent expected colony sizes for an irradiated dose of 2 Gy whereas panels (b), (d) and (f) represent colony sizes for an irradiated dose of 5 Gy.

Looking at panels (e) and (f) it is noticeable that the new model provides two different distributions at Day 5: the distribution on the right hand side peaked at 1024 cells represents those colonies that are almost unaffected and the other one peaked around 64 cells represents severely injured colonies that have a strong time delay. This result suggests that the parameters that have been selected to describe T_{del} are reasonable. At this point a work of refinement of the model has to be done. The first step of the refinement procedure is to understand why distributions still look less broad that those produced with experimental data. This feature is more pronounced at later times, for example at day 3 or day 5, where distributions are well separated in simulations whereas for real data the distributions merge in an intermediate region.

The first fact that has to be taken into consideration is that cells are asynchronous, so from the beginning there is a distribution of cell cycle times: cells can either split earlier or later than 12 h after irradiation. To reproduce this effect a Gaussian distribution centred at 12 h was applied to cell cycle time.

A Gaussian distribution was applied to time delay too. The physical reason why a distribution of time delays is expected is that the same DNA damage can cause very different effects depending on the position where the lesion takes place: an iDSB in a crucial gene is much more severe than an iDSB taking place in a non-coding region of the DNA. As a result the same kind of damage can lead to a variety of time delays. Another reason why to consider a distribution of time delays is that in experimental data there is an uncertainty in the number of cells forming a colony due to the measurement method. This leads to more spread colony size distributions that can be replicated applying a Gaussian to time delay. In Fig. 3.7 two histograms showing time delay at irradiated doses of 1 Gy and 5 Gy are presented. The Gaussian distribution of time delay leads to more spread distributions compared to the previous results in Fig. 3.5.

Various simulations performed with different σ values suggested that the best expression for cell cycle time could be

$$T_{cell} = \frac{1}{\sigma\sqrt{2\pi}}e^{-(x-\mu_{T_{cell}})^2/(2\sigma_{T_{cell}}^2)} \tag{3.7}$$

which is a Gaussian with $\mu_{T_{cell}} = 12$ and $\sigma_{T_{cell}} = 0.7$. Analogously the time delay is described by a Gaussian

$$T_{del} = \frac{1}{\sigma\sqrt{2\pi}}e^{-(x-\mu_{T_{del}})^2/(2\sigma_{T_{del}}^2)} \tag{3.8}$$

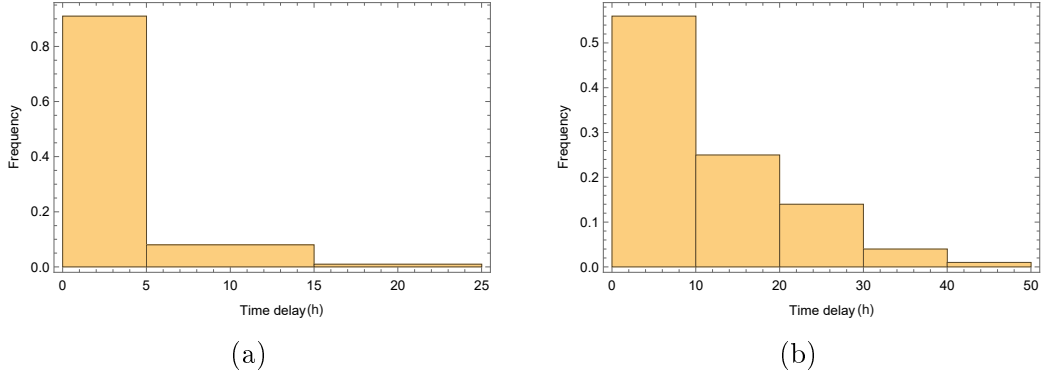(a)   (b)

Figure 3.7: Time delay distributions obtained with Gaussian distributions. Fig. (a) refers to an irradiated dose of 1 Gy whereas Fig. (b) refers to a dose of 5 Gy.

with $\mu_{T_{del}} = c\epsilon_{tot}$ and $\sigma_{T_{del}} = 0.2\mu$. In this case $\sigma_{T_{del}}$ depends on the mean value because, as the total damage increases, many scenarios are possible so time delay spans in a wider range.

To have a deeper analysis of colony sizes, different simulations were made changing the c value and it soon became evident that it was impossible to reproduce both the position of the peaks and the shape of the distributions at the same time in a satisfactory way. As a matter of fact, the constant that gave a better position for the peaks also produced the twice-peaked shape at Day 3 contrary to what happens in real data. The priority was given to the description of the shapes and using constant c=10 the typical distribution with two peaks was present only at day 5 as in the experimental data. With this value for the constant c colony sizes were simulated and some of the resulting histograms are presented in Fig. 3.8. The model reproduces remarkably distributions at early times. Let us take as an example panels (a) and (b) that show colony size distributions two days after irradiation: here both the peaks' positions and distribution's width are similar to experimental results. At day 5 the position of the peaks is not accurate as they appear shifted towards bigger colonies compared to real data. The important aspect though is that this model provides the typical shape with two distributions only at day 5, when it is also present in experimental data.

3.2.1   Survival curves

Survival curves, that represent the amount of cells that are considered as survivors for different irradiated doses, were produced with this model. For
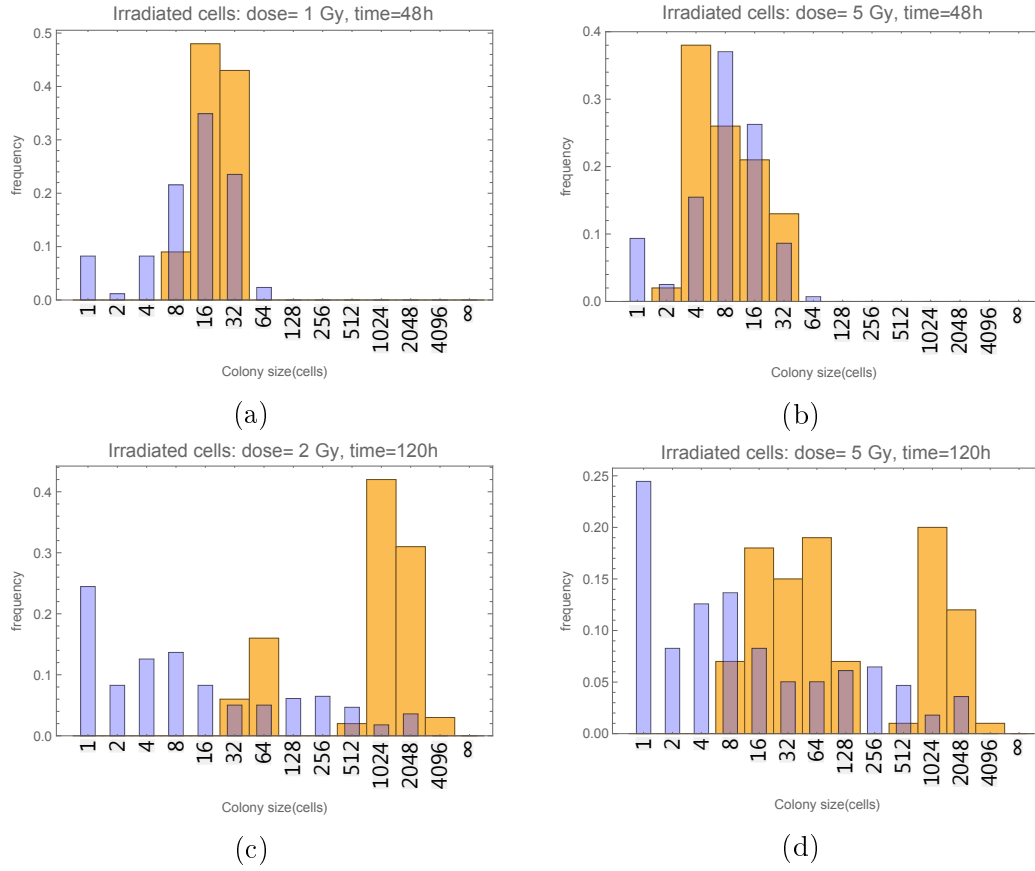
(a)

(b)

(c)

(d)

Figure 3.8: Simulation of colony sizes 2 and 5 Days after irradiation performed with the Gaussian distributed time delay. Panels (a) and (b) present the results expected 48 hours after irradiation respectively for irradiated doses of 2 Gy and 5 Gy. Panels (c) and (d) present the results at 120 hours after irradiation respectively for irradiated doses of 2 Gy and 5 Gy.

the first analysis the threshold criterion to select surviving colonies was set to 50 cells. In order to have higher statistics and more precise fits an initial amount of a thousand cells was taken. Survival curves were simulated in a time span between two and seven days after irradiation. For a direct comparison with the LQ model, α and β values were obtained using the following equations

$$\alpha = \epsilon_i \, \alpha_{DSB} \qquad (3.9)$$

$$\beta = \frac{\epsilon_c}{2} \frac{\alpha_{DSB}^2}{N_L} - \alpha \frac{\alpha_{DSB}}{N_L} \qquad\qquad (3.10)$$

derived by Friedrich et al.[2] As explained in section 2.1.1, α_{DSB} is the initial yield of DSB and N_L is the number of chromatine loops contained in the genome. Replacing those parameters in equation 1.1 the survival curve for the linear quadratic model was obtained. This curve is represented in black in the following graphs.

In Fig. 3.9 the results are presented. Panels (a) and (b) refer to a first analysis performed with $\sigma_{T_{del}} = 0.7\,\mu$. The high $\sigma_{T_{del}}$ value led to survival curves that were not close to the linear-quadratic black curve. In these graphs it is also noticeable that below 10 % survival level simulated data drift from the linear quadratic curve. This is an artificial effect due to the Gaussian tails that cause a comparably high number of surviving cells at high doses and low survival levels. A possibility to solve the problem is to use a lower value for μ so that simulated data drift from the linear quadratic curves at high doses and low survival levels and do not affect the analysis. Panels (c) and (d) were produced setting $\sigma_{T_{del}}$ to the value of $0.2\,\mu$ that gives survival curves more similar to what is predicted by the linear quadratic model. To further improve the analysis Gaussian distributions that had been applied to cell cycle time and to time delay were cut at 3 σ.

Simulated data were then fitted with a curve with linear quadratic dependence of the dose, such as $y = -\alpha x - \beta x^2$ where y is the fraction of surviving cells and x is the dose. With this procedure the α and β values were derived to have a direct comparison to those given by the linear-quadratic model. The α and β values are plotted in Fig. 3.10 against time. These plots only take into consideration those values obtained from 80 h on, because of the survival criterion: 50 cells colonies are only present 60-70 h after irradiation so fits do not produce reliable results before that time. The α value, that describes the initial slope of survival curves, decreases at increasing times. Negative α values do not have a physical meaning, they are due to the fitting procedure to simulated data. The β parameter is almost constant in the whole time range.
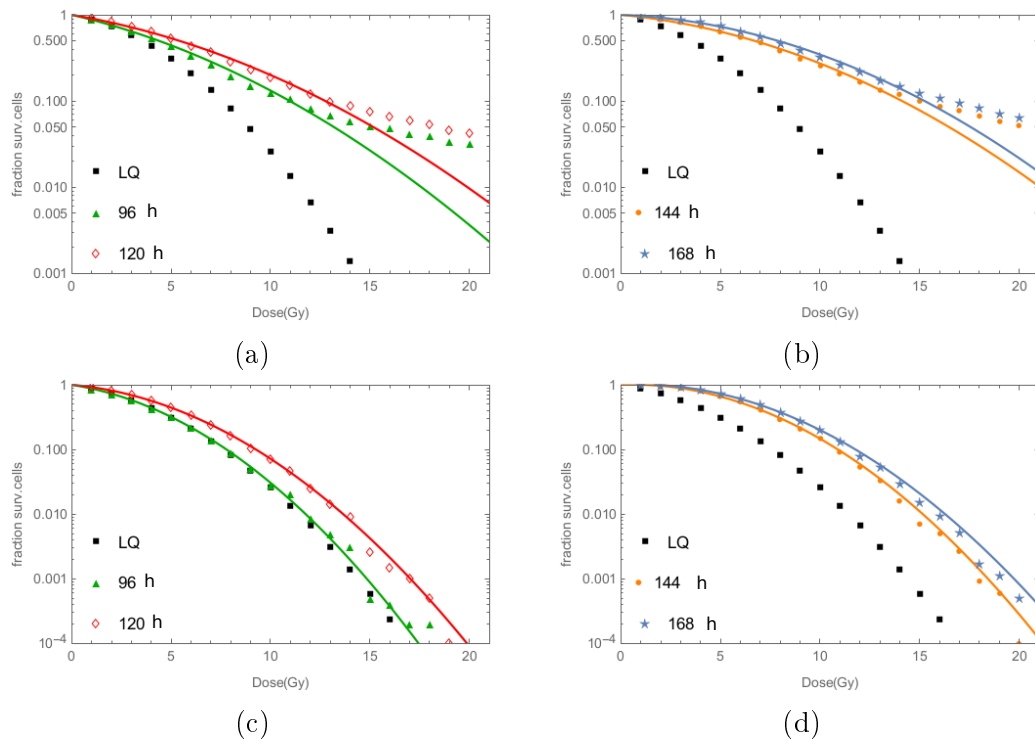
(a)

(b)

(c)

(d)

Figure 3.9: Simulated survival curves. Fig. (a) and (b) were obtained setting $\sigma_{T_{del}} = 0.7\,\mu$. Fig. (c) and (d) were obtained setting $\sigma_{T_{del}} = 0.2\,\mu$ instead. Different colours correspond to different times after irradiation. Simulated data have been fitted with a linear-quadratic curve to allow a comparison with the LQ model.
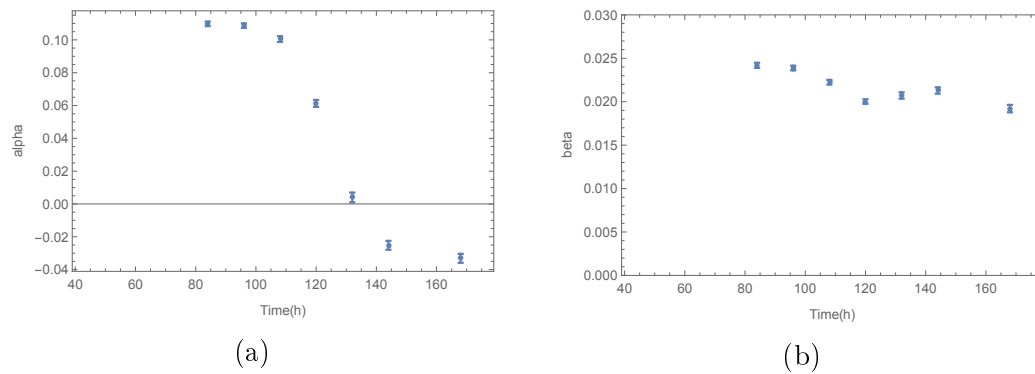

(a)

(b)

Figure 3.10: In these graphs α and β values, derived from the fit to survival curves, are plotted against time.

3.2.2   Fit ranges

To prove the robustness of the analysis of survival curves, different fits were performed, taking into consideration different lower values for the survival fraction. In Fig. 3.11 panel (a) and panel (b) show two fits to the same data set performed considering a lower survival fraction of respectively 1% and 1 ‰. Panels (c) and (d) show the correspondent α values at increasing times.
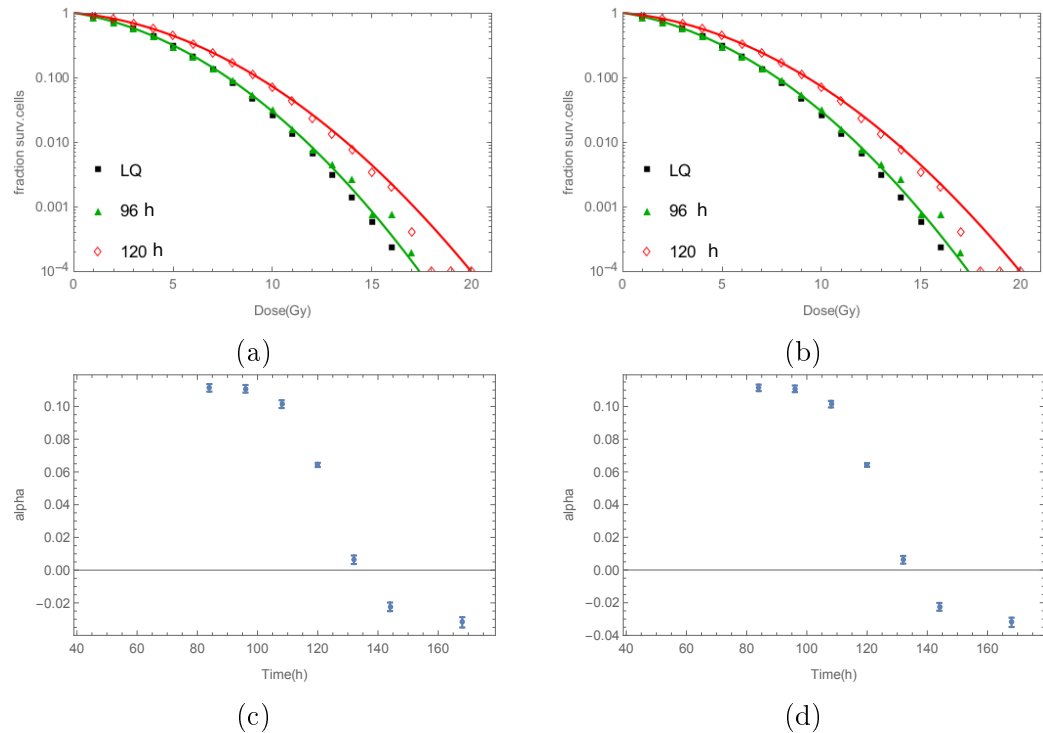

Figure 3.11: On top two simulations of survival curves are presented. In (a) and (b) simulated data have been fitted with linear quadratic curve considering different y ranges: in (a) the lowest survival fraction was set to 1 % whereas in (b) it was set to 1 ‰. The corresponding α values are plotted in panels (c) and (d).

Since there is no appreciable difference between the two fitting methods, the lower survival level was set to 1% according to what can be found in literature. In most of the survival curves data situated at higher doses are not in good agreement with the linear quadratic fitted curve, so another aspect that has been checked is the role of the dose range considered for the fit. Some fits were performed using three different dose ranges: from 0 to 5 Gy, from 0 to 10 Gy and from 0 to 20 Gy. In Fig. 3.12 α and β have been

obtained fitting respectively from 0 to 10 Gy for panels (a) and (b) and from 0 to 20 Gy for panels (c) and (d). Those final graphs again are very similar to each other so it can be concluded that the dose range does not have a strong impact on α and β. Since the dose range does not seem to affect much the final parameters, fits can be performed in the whole dose range.
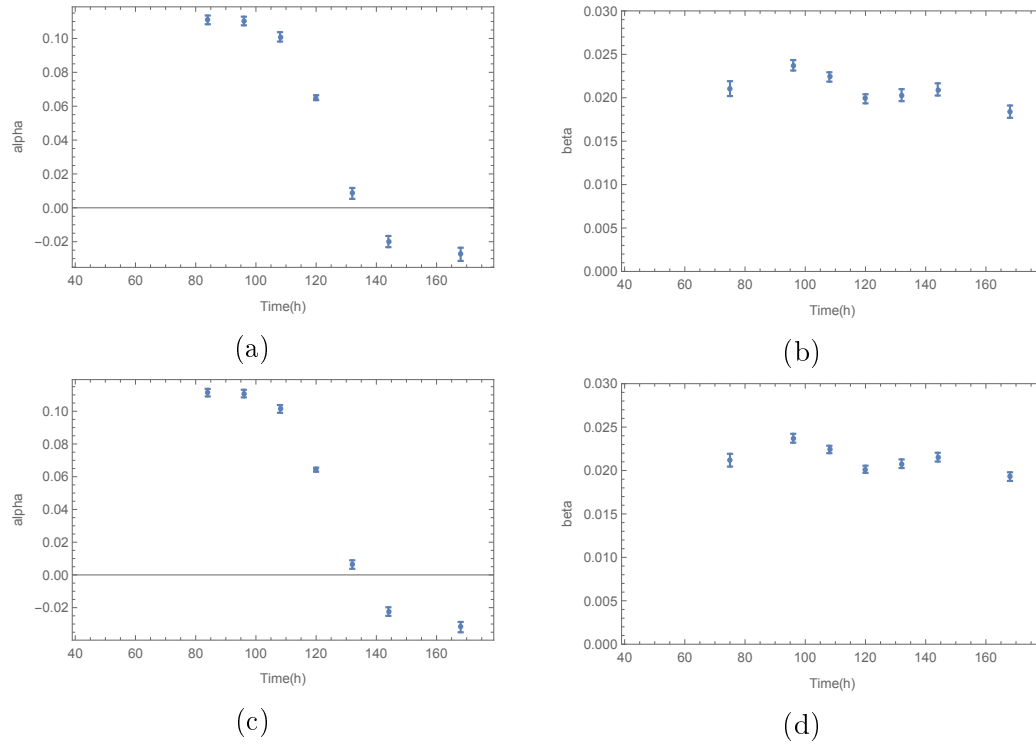

(a)

(b)

(c)

(d)

Figure 3.12: Different dose ranges have been considered for the fits to survival curves. Panels (a) and (b) present the α and β values obtained fitting survival curves in a range from 0 o 10 Gy. Panels (c) and (d) present the α and β values obtained fitting in the entire dose range.

3.2.3 Survival criteria

An important feature of this work was to determine the threshold criterion to discriminate surviving colonies from non surviving colonies and to see how different criteria could affect the expected fraction of surviving cells. In order to do this the analysis was repeated using different threshold criteria to select surviving colonies.
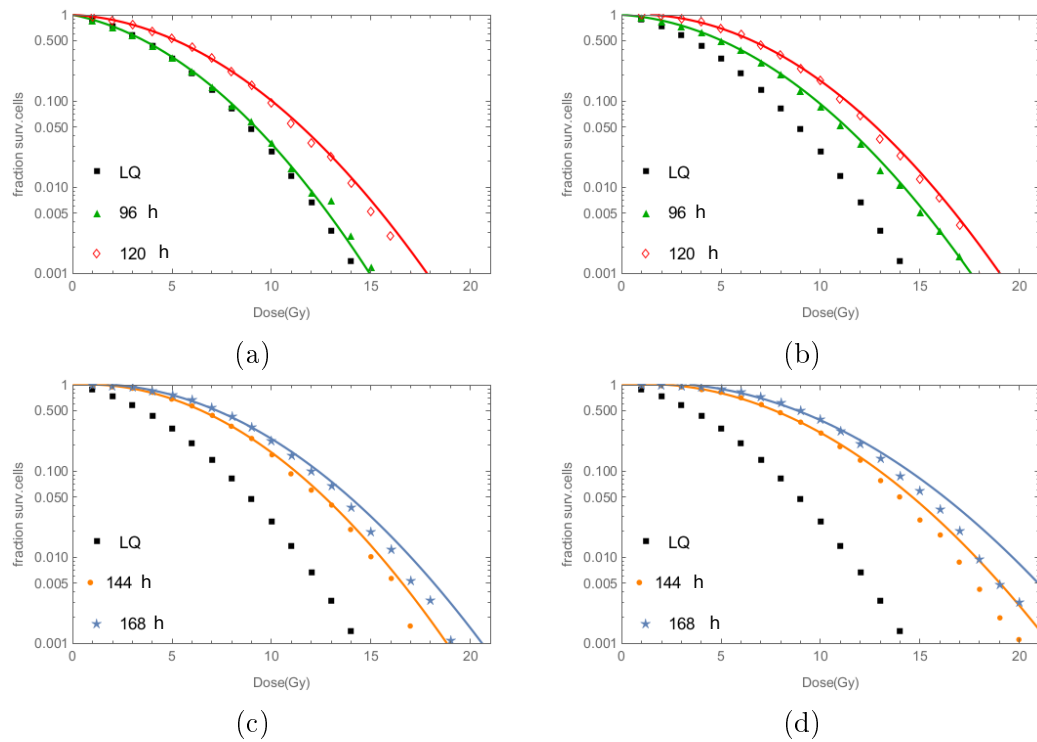
Figure 3.13: Some representative survival curves obtained using different thresholds to discriminate surviving colonies. A threshold of 40 cells was used to produce graphs (a) and (c) that represent survival curves at different times. The threshold was set to 20 cells in graphs (b) and (d).

Representative survival curves are shown in Fig. 3.13. In particular there is a comparison between survival curves obtained considering 40 cells as threshold criterion in panels (a) and (c), and 20 cells threshold criterion in panels (b) and (d). The overall effect caused by a lower survival criterion is a shift of survival curves towards higher y values. Even if survival curves and their corresponding linear-quadratic fits obtained with different threshold criteria are different, the α and β values are not strongly affected. As an example in Fig. 3.14 α and β were obtained setting the threshold criterion to 20 cells. It is visible that the two parameters vary in the same range as in Fig. 3.10 where the threshold criterion was set to 50 cells. There is no hint that α and β should change applying this growth model to different cell lines, considering that the cell cycle time and the lethalities should be adapted to the new cell type. With reference to the article of F.Gaun,[1] the

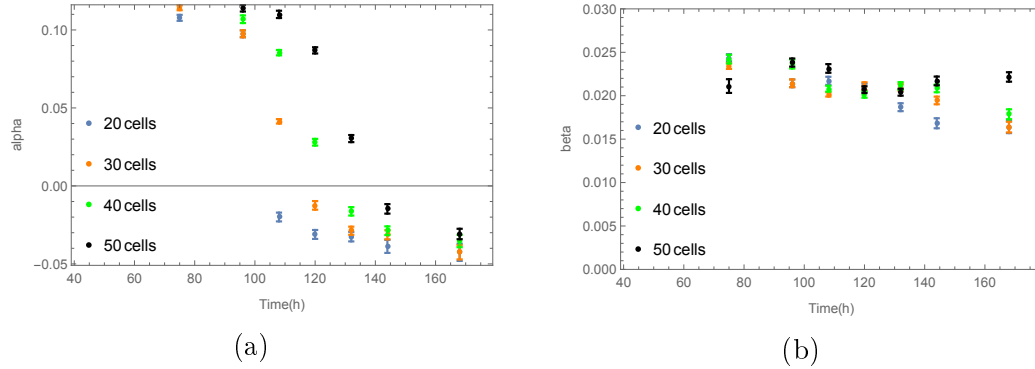colony growth measurements performed 5 Days after irradiation with the 50 cells threshold criterion should give reliable values for α and β parameters.

Figure 3.14: In this figure a comparison between α and β obtained with different threshold criteria is presented. Each colour represents a different criterion,which is reported in the legend in terms of threshold number of cells.

3.2.4 Growth curves

One important aspect of the thesis was to produce growth curves and to compare them to the ones in the work of G.Böhrnsen. In Fig. 3.15 (a) simulated and experimental growth curves are presented. Graph 3.15 (a) was produced using experimental data relative to an irradiated dose of 5 Gy. All curves start from a value of one cell which corresponds to time 0. Then curves grow exponentially until a time of around 50 hours after irradiation. This happens because even if cells are damaged, most of the times they undergo a few cell cycles before loosing their reproductive integrity so at early times colony growth can proceed almost regularly. After 50 hours some colonies keep growing exponentially but some others show a saturation to a constant number of cells. The saturation is due to mutations and lesions within cells that prevent cells from splitting. This can happen to the whole colony or only to some cells and this is why colonies go to saturation at different times, reaching different final colony sizes. A few curves drop to zero, this means that the colony was not measurable either because it grew in the borders of the flask not allowing a precise measurement of the occupied area or because dead cells decomposed so the colony actually "disappeared".

In Fig. 3.15 (b) growth curves have been simulated for an initial set of 100 cells and for an irradiated dose of 5 Gy. The graph is characterized by

three different groups of cells. The first group at the top represents those
cells which grow almost without any time delay also referred to as unaffected
cells. Then in the middle there is a group of curves that represents all those
cells for which the total effect equals to one. The last group in the bottom
represents all the cells characterized by a total effect higher than one. The
main lack of the model is that simulated curves grow exponentially at every
time so all the curves that in real data go to a constant colony size are not
represented. A solution to this problem will be proposed in the next section.
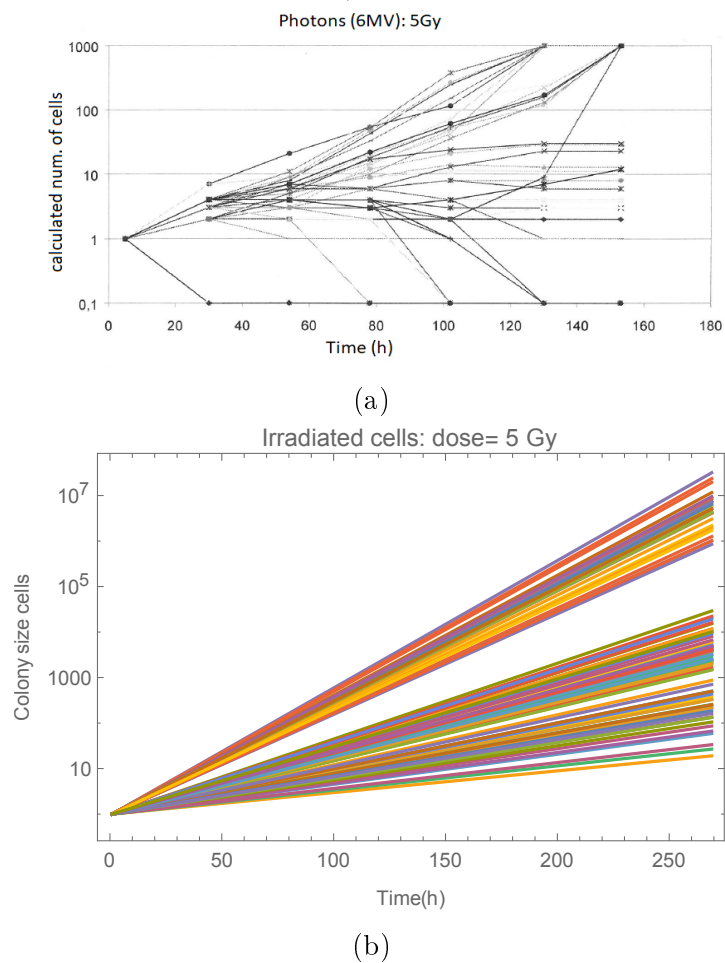

(a)

(b)

Figure 3.15:   Comparison between experimental
growth curves (a) and simulated cell growth (b). In
both figures the irradiated dose corresponds to 5 Gy.

3.3 Final refinements

The final refinement of the model was to find a proper way to reproduce colony growth curves as they appear in real data. In order to do that the proposed solution was to redefine the expected number of cells at time t with a new equation:

$$N_{cells}(t) = 2^{t/[T_{cell}+T_{del}+f_2\epsilon_{tot}^{f_1}/(f_3(T_{cell}+T_{del}))]} \tag{3.11}$$

where T_{cell} is, as usual, the cell cycle time, T_{del} is the time delay, ϵ_{tot} is the total effect and f_1 ,f_2, f_3 are arbitrary constants. Comparing various simulations performed with different combinations of f constants, the values that gave a better representation of experimental results were $f_1 = 2.5$, $f_2 = 1$ and $f_3 = 6$.

The definition of this new equation does not affect the distribution of time delay, so the results presented in Fig. 3.7 are still valid. All the analysis of colony sizes and survival curves is on the contrary affected by this new equation for N_{cells}, thus the analysis was repeated one last time. In order to allow a comparison with real data the lethalities ϵ_i and ϵ_c were set, as in the previous analysis, respectively to 0.0036 and 0.18.

3.3.1 Colony sizes

Colony sizes were simulated considering an initial amount of 100 cells and using equations 3.7 and 3.8 to derive cell cycle time and time delay.

Four representative histograms are shown in Fig. 3.16. Colony size distributions presented in each panel are slightly different from the ones obtained with the previous model, presented in Fig. 3.8. As before, simulated distributions fit well experimental data at low doses and early times but they become more imprecise at later times. At day 5 for example peaks are not placed in the same position as in real data, but the important feature which is reproduced by the model is the presence of two distributions in simulations whenever they appear in experimental measurements too. Once verified that the model fulfilled the main requirements concerning the reproduction of colony sizes, it was possible to proceed with the other steps of the analysis.

3.3.2 Survival curves

Survival curves were then produced using this new model. As before, a thousand cells were considered as initial group in order to have less uncertainties and as a consequence better fits.
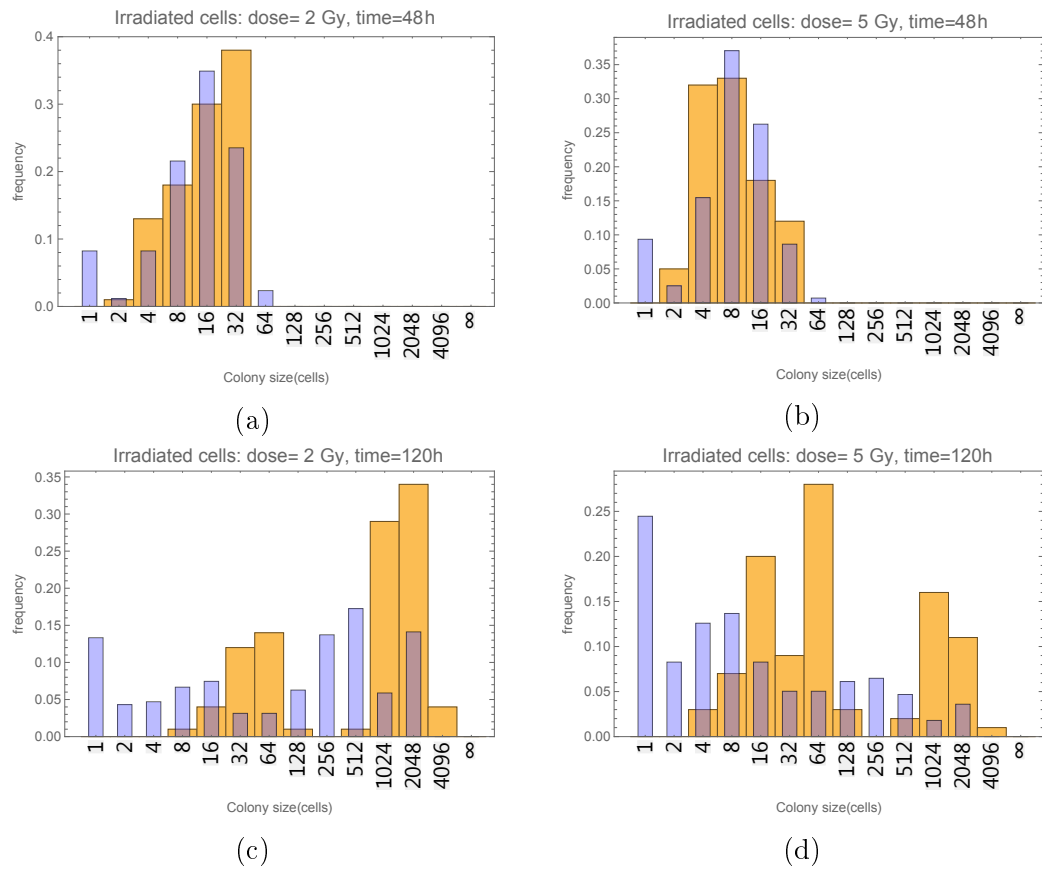
(a)

(b)

(c)

(d)

Figure 3.16: Simulation of colony sizes 2 and 5 Days after irradiation using eq. 3.11 to derive the number of cells forming colonies in the growth fase. Panels (a) and (b) present the results expected 48 hours after irradiation respectively for irradiated doses of 2 Gy and 5 Gy. Panels (c) and (d) present the results at 120 hours after irradiation respectively for irradiated doses of 2 Gy and 5 Gy.

Some survival curves are presented in Fig. 3.17. The green curve represents cell survival after 96 hours and it is almost overlapped to the linear quadratic prediction, for which the α and β parameters were obtained through equations 3.9 and 3.10. Survival curves simulated at later times are shifted towards higher survival fractions with respect to the green curve but from 144 hours on there is no more distinction between survival curves taken at different times and they appear all overlapped. This means that after 144 hours the situation is quite stable and the amount of colonies that exceed

the 50 cells criterion at a fixed irradiated dose will be the same considering different times after irradiation. This result is in agreement with the usual procedure for colony size measurements, that is performed one week after irradiation. Panels (c) and (d) in Fig. 3.17 show the parameters derived from the fit to survival curves. The results are very similar to the ones obtained using the previous model (Fig. 3.10). In fact α decreases with increasing time whereas β is almost constant at all times.
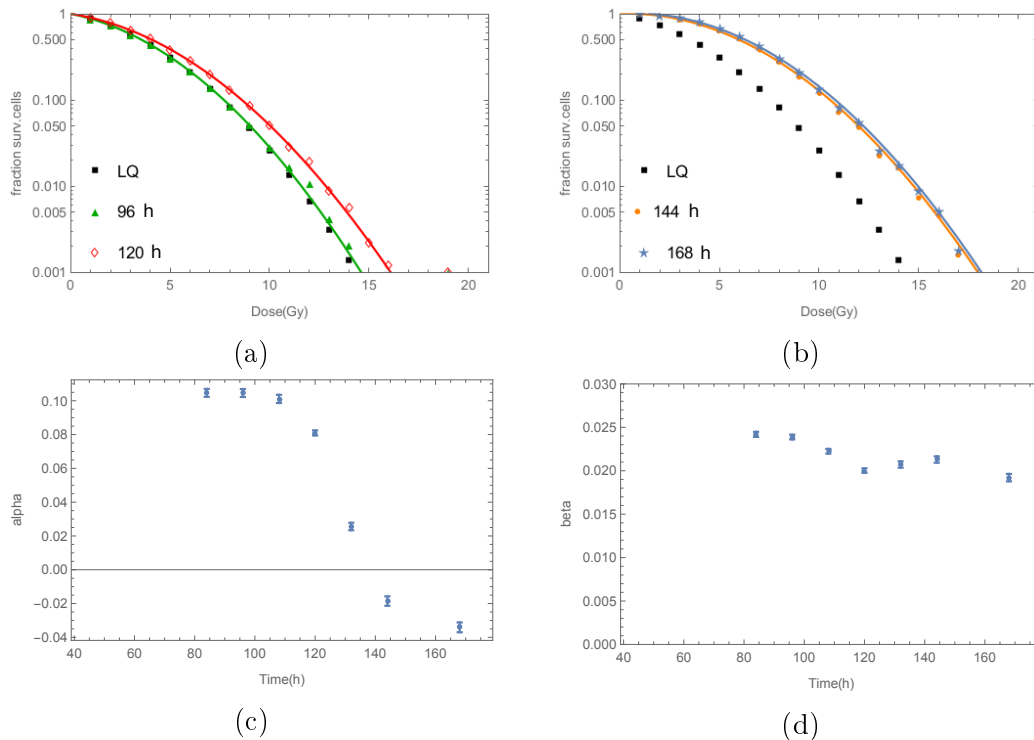

Figure 3.17: Two representative survival curves are presented in (a) and (b). Different colours correspond to different time lapses after irradiation. The α and β parameters derived from survival curves α are respectively presented in panels (c) and (d).

Again different fit ranges were tested to assess if the results could be affected by a different choice of dose range or survival range where to perform fits. The analysis led to similar results to what is presented in section 3.2.3, so fits were in the end performed in the whole dose range and setting the lower limit for the fraction of surviving cells at 1 %.

3.3.3   Threshold criteria

Different threshold criteria to discriminate surviving colonies from non sur-
viving ones were tested for this model in order to verify the robustness of the
previous analysis. Some of the produced graphs are presented in Fig. 3.18.
In this case the threshold criterion was set to 20 cells so it was possible to
include also data simulated at 60 hours. The green curve in this case is not
overlapped to the black curve predicted by the LQ model and there is also a
difference between the curves taken at later times that were overlapped using
the 50 cells criterion. This is caused by the fact that colonies affected by a
strong time delay might exceed 20 cells even at later times without reach-
ing a size of 50 cells. Although survival curves are different based on what
criterion is used, panels (c) and (d) in Fig. 3.18 show that α and β do not
differ much from the results obtained using the 50 cells criterion. It can be
concluded that different criteria cause a shift in survival curves but do not
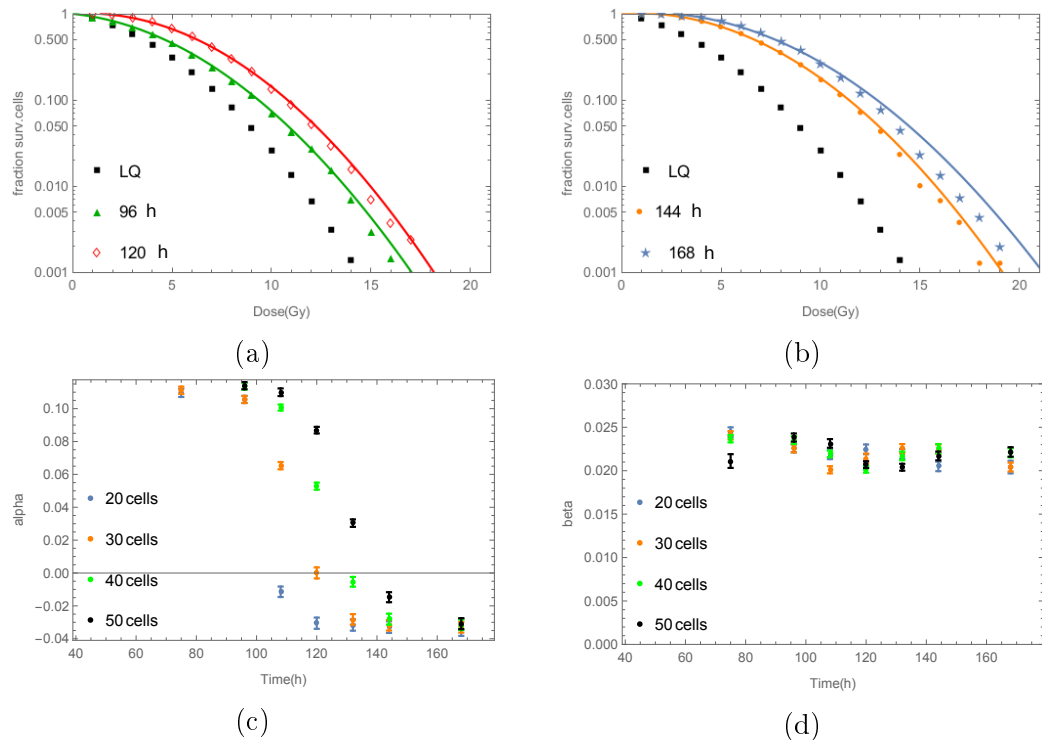affect much the linear quadratic parameters.

Figure 3.18: In this figure panels (a) and (b) show sur-
vival curves obtained setting the threshold criterion to
20 cells. Panels (c) and (d) show α and β obtained
with different threshold criteria.

3.3.4 Growth curves

The last step of the analysis and the main reason why the new model was introduced, was the production of growth curves. A set of a hundred initial cells was taken and four graphs were produced considering irradiated doses of 1 Gy, 2 Gy, 3 Gy and 5 Gy. The resulting plots are shown in Fig. 3.19. At a dose of 1 Gy (panel (a)) most of the curves grow exponentially because there are only a few lethal events so cells most of the times can continue their normal cell cycle. There is a group of curves that show a delay in their growth so these colonies are smaller then the unaffected ones. Panel (b) refers to an irradiated dose of 2 Gy. Here more colonies tend to show a delayed growth and within those colonies two subgroups can be distinguished: the subgroup in the middle of the graph describes those initial cells for which the total effect equals to one, whereas the group at the bottom contains those cells characterized by a total effect equal to 2. A similar distinction can be done in graph (c) where in addition there are a few curves, in a lower position than the second group, that are characterized by a total effect of 3. In graph (d) a third group can be clearly distinguished at small colony sizes and it is composed by those cells for which the total effect is bigger than 2. Looking at simulations ϵ_{tot} can reach a maximum value of 6 at an irradiated dose of 5 Gy. Considering the results of the simulations a comparison can be done with experimental growth curves. Looking at Fig. 3.20 control cells have a similar representation in simulations and in experimental data. All curves grow exponentially with slightly different slopes due to cell cycle time Gaussian distribution. At 5 Gy several colonies grow with an appreciable time delay and the curves situated in the lower region of the graph do not even reach a thousand cells after 200 hours. The values of f_1, f_2 and f_3 in Eq. 3.11 affect especially the slope of those curves that represent cells with a strongly delayed growth. Usually colony size assays are performed 7 days after irradiation and the threshold criterion used to discriminate survivors from non survivors is 50 cells. The choice of the parameters f_1, f_2 and f_3 was made so that all the colonies that would survive should be bigger than 50 cells already at 168 hours. All the colonies that have a strong time delay and as a consequence are smaller than 50 cells at 168 h after irradiation should not exceed 50 cells even if measured at a later time.

With this model and this specific choice of all parameters it was finally possible to give a better description of colony growth after irradiation. Colonies affected by a strong time delay are described by curves with smaller slopes and colonies that are scored as dead after 168 hours do not exceed 50 cells for a long time lapse.
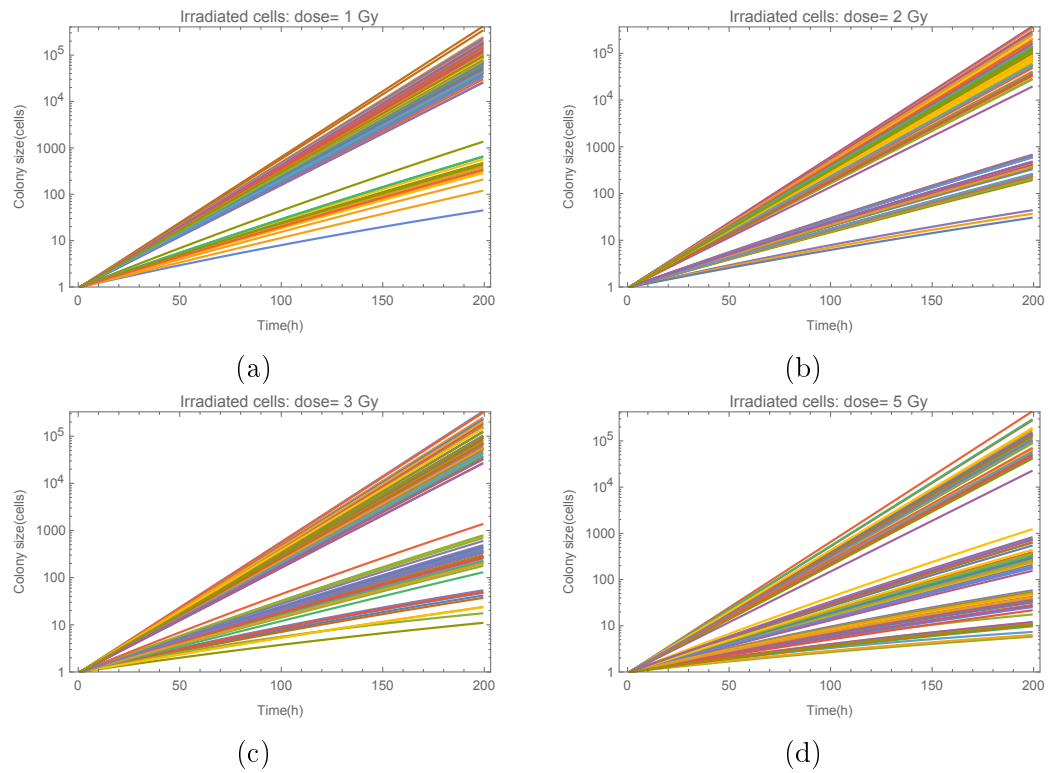
Figure 3.19: This figure presents the final simulation of growth curves. In each panel a different irradiated dose is considered.
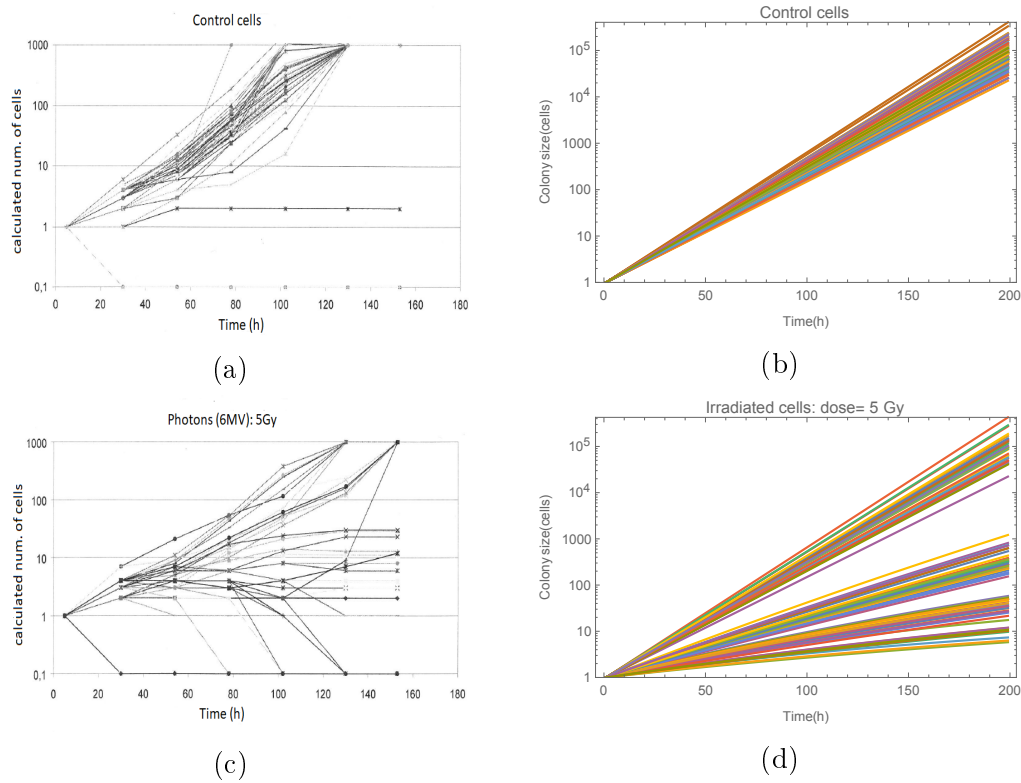
(a)

(b)

(c)

(d)

Figure 3.20: Comparison between experimental growth curves in (a) and (b) and simulated growth curves in (c) and (d). The two pictures on top refer to control cells whereas the graphs on bottom refer to an irradiated dose of 5 Gy.

Chapter 4

Conclusions and Perspectives

In this work a model to describe colony growth after irradiation was developed. Looking at the results presented in 3.3 it is possible to conclude that the time delay observed in colony growth after irradiation depends on the total effect due to both isolated and clustered double strand breaks. The idea that allowed to better reproduce experimental colony sizes was to derive this total effect taking random numbers from a single Poissonian distribution with mean $n_i\,\epsilon_i + n_c\,\epsilon_c$ using n_i and n_c values obtained with GLOBLE model. This different method lead to more spread colony size distributions. The description that has been proposed for time delay can reproduce well colony size distributions especially at low doses. When higher doses are used the model reproduces the shape of colony size distributions but it does not give a precise position for the peaks. From the simulated survival curves α and β parameters were derived with a fitting procedure. Of particular interest is the fact that β is almost constant in time for every irradiated dose in the range of 0 to 5 Gy. The α parameter decreases with increasing time instead. The fitting method has been tested using different dose ranges and different survival ranges. The estimate of the parameters was not appreciably affected by the choice of different ranges where to perform the fit.

The analysis was repeated using different threshold criteria to define colony survival. Plots of α and β against time have been produced using threshold criteria in a range from 20 to 50 cells. It can be concluded that the threshold criterion does not affect much the final parameters α and β. With reference to the article by F. Guan et al.,[1] it can be said that colony size measurements taken at earlier times than one week after irradiation should not produce different values for α and β. The analysis has been performed referring specifically to V79 cells but there is no hint that the overall behaviour of α and β should change taking in consideration different cell lines. According to the specific cell line proper values for lethalities should be used to derive

the total effect and the equation describing colony growth should be adapted considering the correct value for cell cycle time. In order to simulate growth curves a new equation has been introduced to describe the expected number of cells forming a colony at a time t after irradiation. The resulting plots of colony size expressed in terms of number of cells against time reproduce some features that can be observed in real data. Especially at an irradiated dose of 5 Gy growth curves that represent severely damaged cells show a smaller slope and produce smaller colonies compared to unaffected cells. Another interesting feature is that cells characterized by different values for the total effect ϵ_{tot} are distinguishable in plots that represent colony size against time.

Many different studies and experiments have been performed through the years with the aim of describing cell growth after irradiation. Although results obtained under different conditions cannot be directly compared, it is interesting to notice that some aspects discussed in this thesis are consistent with what can be found in a few articles that present specific measurements of the number of cells within a colony. As an example Dewey et al.,[10] in 1963, irradiated mammalian cells with photons and studied the resulting inhibition of cellular multiplication and colony formation. In particular L-P59 cells, characterized by a cell cycle time of 24 hours, were irradiated with different doses going from 0 Gy to 17.5 Gy. At an irradiated dose of 10 Gy, colonies showed a delayed growth and their estimated cell cycle time was 44 hours. Although the results obtained in this work of thesis cannot be directly compared to the experimental data, a simulation run with GM3 at 10 Gy, for V79 cells, gives a distribution of time delays averaged at 37 hours. The simulated time delays at 10 Gy is consistent with what was reported by Dewey in his work.

An article published by Sinclair et al. in 1964[11] studied the response of hamster cells to photon irradiation. In particular the authors presented measurements of colony size distributions taken 13 days after X-Rays irradiation. Colony size distributions appear narrow for control cells and for those cells that were irradiated with low doses, but they become broader as the irradiated dose increases, in agreement with what was obtained in this work of thesis. The results presented in this article do not offer a complete overview of colony size distributions because colony assays were only taken 13 days after irradiation. Another interesting feature was remarked by the authors: the relative fraction of small colonies increases with increasing doses. This is consistent with what is presented in Fig. 3.8, where it is visible that the fraction of small colonies increases at higher irradiated doses.

In 1989 D.Bettega et al.[12] investigated the growth kinetics of mouse embryo cells exposed to proton irradiation in a dose range from 0 to 7 Gy. The authors found that the fraction of small colonies was bigger for increasing

irradiated doses, a feature that is reproduced by GM3 model.

In this article there is a comparison between two survival curves at 100 hours and at 10 days after irradiation. The first curve represents the surviving fraction of cells measured with the 50 cells criterion 10 days after irradiation, the second curve represents the fraction of colonies that had at least 3 cell divisions after 100 hours. Survival curves are not affected by the choice of different criteria, and this is consistent with the analysis presented in this work, where the α and β parameters derived by survival curves obtained with GM3, are not remarkably affected by the use of different threshold criteria.

To explain experimental results the authors proposed a mathematical model that assumes that after irradiation two subpopulations can be distinguished within a colony. The first subpopulation is formed by surviving cells and the second one by non surviving cells. This model does not distinguish between unaffected cells and those cells that are affected by irradiation but can still divide, with a time delay in their cell cycle. The authors concluded that the behaviour of growth curves is affected by non surviving cells until around 150-200 hours after irradiation, depending on the irradiated dose, and growth curves grow exponentially after that time lapse. The results obtained is this thesis suggest on the contrary that growth curves are affected by severely injured cells even after a long time.

There are also some recent papers that investigate colony size distributions after irradiation. For example T. Sakashita at al.[13] studied colony size distributions for those colonies that did not survive after irradiation. The experiments were performed with normal human fibroblasts, irradiated with photons up to 8 Gy. An increase of the fraction of small colonies with increasing irradiated dose was reported. Experimental data were analysed using a simple branching process model analysis that only allowed to reproduce small colonies (~ 20 cells). Even if the models and methods used in this thesis are different from the ones adopted by Sakashita et al. for their analysis, it is noticeable that the fraction of small colonies increases at higher doses in both cases.

An interesting future perspective to extend the analysis performed in this work, would be to use high-LET irradiation and to study its impact on colony growth. In this case the GLOBLE model cannot be used to derive lethalities because high LET radiation is not homogeneously distributed so the basic assumptions of the model are not fulfilled. The LEM model[6–8] can be used instead to obtain the lethalities ϵ_i and ϵ_c associated to isolated and clustered double strand breaks. Using these values the whole analysis that has been performed in this work could be repeated and colony growth curves could be produced.

A starting point for the validation of the analysis with high-LET irradi-

ation could be a comparison with the results published by Sakashita et al.[14] In this paper the authors investigate the impact of carbon ions irradiation on colony size distributions and on RBE. Different criteria were chosen to discriminate survivors from non survivors, setting the initial threshold at 10 cells and then increasing its value in steps of 10 until a the value 100 was reached. The conclusion was that different threshold criteria did not affect significantly the RBE value. Survival curves obtained using low survival criteria do not reproduce well the expected exponential relation between the surviving fraction of cells and the irradiated dose. As a result the values obtained with a fitting procedure to those graphs cannot be considered as reliable. This is the reason why this article could only be used for a first comparison with simulations but it would not be sufficient for a complete validation of the growth model proposed in this thesis.

If the model will be able to describe cell growth for ion and photon irradiation some other improvements could be done in order to have a more precise analysis.

For example the analysis could be repeated for different cell lines. Colony size measurements could be performed in parallel in order to allow a direct comparison with simulated data. The resulting values of α and β are expected to be similar to what has been obtained for V79 cells. The second improvement would be to find a better description for growth curves. The goal would be to describe completely colony growth only modifying the time delay equation, rather then introducing a new description for the expected number of cells. Although these final corrections can be helpful in order to have a more precise reproduction of growth curves and a more complete analysis, they are not expected to have a strong impact on survival curves and consequently on α and β.

Acknowledgements

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