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Bioluminescence: Fundamentals and Applications in Biotechnology–Volume 2

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This book series reviews current trends in modern biotechnology and biochemical engineering. Its aim is to cover all aspects of these interdisciplinary disciplines, where knowledge, methods and expertise are required from chemistry, biochemistry, microbiology, molecular biology, chemical engineering and computer science.

Volumes are organized topically and provide a comprehensive discussion of developments in the field over the past 3–5 years. The series also discusses new discoveries and applications. Special volumes are dedicated to selected topics which focus on new biotechnological products and new processes for their synthesis and purification.

In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

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Editors

Bioluminescence: Fundamentals and Applications in Biotechnology–Volume 2

With contributions by

Elizabeth Anderson · M. Angeles Muñoz-Martín
Shimshon Belkin · Tom Ellis
Francisca Fernández-Piñas · Paul Freemont
Miguel González-Pleiter · Matti Karp
Francisco Leganés · Arne Lundin · Benjamin Reeve
Ismael Rodea-Palomares · Theo Sanderson
Vyv Salisbury · Nina Virolainen · Sharon Yagur-Kroll

Springer

Editors
Gérald Thouand
UMR CNRS 6144
University of Nantes
France

Robert Marks
Department for Biotechnology Engineering
Ben Gurion University of the Negev
Beer Sheva
Israel

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Contents

Part I Fundamentals of Bioluminescence

How Synthetic Biology Will Reconsider Natural Bioluminescence and Its Applications 3
Benjamin Reeve, Theo Sanderson, Tom Ellis and Paul Freemont

Optimization of the Firefly Luciferase Reaction for Analytical Purposes 31
Arne Lundin

Part II Applications of Bioluminescence in Environment and Security

Evaluation of the Ecotoxicity of Pollutants with Bioluminescent Microorganisms 65
Francisca Fernández-Piñas, Ismael Rodea-Palomares, Francisco Leganés, Miguel González-Pleiter and M. Angeles Muñoz-Martín

Molecular Manipulations for Enhancing Luminescent Bioreporters Performance in the Detection of Toxic Chemicals 137
Sharon Yagur-Kroll and Shimshon Belkin

Part III Applications of Bioluminescence in Agriculture and Bioprocess

Biosensors, Antibiotics and Food 153
Nina Virolainen and Matti Karp

Part IV Applications of Bioluminescence in Health

Rapid In-vitro Testing for Chemotherapy Sensitivity in Leukaemia Patients 189
Elizabeth Anderson and Vyv Salisbury

Index 215

Part I
Fundamentals of Bioluminescence

How Synthetic Biology Will Reconsider Natural Bioluminescence and Its Applications

Benjamin Reeve, Theo Sanderson, Tom Ellis and Paul Freemont

Abstract As our understanding of natural biological systems grows, so too does our ability to alter and rebuild them. Synthetic biology is the application of engineering principles to biology in order to design and construct novel biological systems for specific applications. Bioluminescent organisms offer a treasure trove of light-emitting enzymes that may have applications in many areas of bioengineering, from biosensors to lighting. A few select bioluminescent organisms have been well researched and the molecular and genetic basis of their luminescent abilities elucidated, with work underway to understand the basis of luminescence in many others. Synthetic biology will aim to package these light-emitting systems as self-contained biological modules, characterize their properties, and then optimize them for use in other chassis organisms. As this catalog of biological parts grows, synthetic biologists will be able to engineer complex biological systems with the ability to emit light. These may use luminescence for an array of disparate functions, from providing illumination to conveying information or allowing communication between organisms.

Keywords Bio-lighting · Bioluminescence · Chassis organisms · Synthetic biology

Contents

1 Introduction 4
2 Applications 5
2.1 Illumination 5

B. Reeve · T. Ellis · P. Freemont (✉)
Centre for Synthetic Biology and Innovation, Imperial College London, London SW7 2AZ, UK
e-mail: p.freemont@imperial.ac.uk

T. Sanderson
The Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK

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2.2 Aesthetics 6
2.3 Communication with Humans 7
2.4 Communication with Other Engineered Systems 8
3 Feasibility of Bio-lighting 9
3.1 Photosynthetic Efficiency 9
3.2 Energy Output 9
3.3 Competing with Conventional Lighting 10
4 Bioluminescent Parts and Modules in Nature 12
4.1 Firefly Luminescence 12
4.2 Bacterial Bioluminescence 15
4.3 Other Natural Luminescence Systems 17
5 Selecting Chassis Organisms 18
6 Controlling Luminescence 22
7 Containment 23
8 Improving and Altering Luminescence 26
8.1 Alterations 27
9 Conclusions 28
References 28

1 Introduction

Bioluminescence in nature has a surreal beauty that captures the imagination of scientists and the public alike. In the laboratory, bioluminescence has been central to techniques in biochemistry and molecular genetics for many years. The young field of synthetic biology has dabbled with bioluminescence, which looks set to be a part of many emerging technologies in this area, both in the near and more distant future.

Synthetic biology aims to design and model novel biomolecular components, networks, and pathways, which are then applied to rewire and reprogram organisms to provide solutions for various challenges [26]. Engineered biological systems might use light emission for a variety of purposes. Proposed applications cover many areas of human activity, from bioluminescent trees for lighting to the use of light for cell-to-cell communication. Each application will require differently optimized bioluminescent systems.

The natural bioluminescent world includes such varied creatures as beetles, fungi, plankton, and bacteria. To the synthetic biologist, this is an archive from which enzymes with desired properties can be selected. The engineer must then aim for a synergy between the bioluminescent parts, other genetic elements drawn from other parts of nature, and the biological ‘chassis’ in which they are all housed. Sometimes natural parts may need to be re-engineered to better suit the chassis or the purpose for which the organism is intended. The designer must also consider the resultant genetically modified organism (GMO) and any risk it might pose to the environment. It may be possible to mitigate any risks by building in biological and genetic containment systems.

Synthetic biology employs modularization, which is intended to allow any bioluminescent module to be used in a wide variety of contexts. Through the use of different promoters, luminescence could be induced by different environmental conditions or, through more complex approaches, tight spatial and temporal control may be possible.

Through characterization and modelling, synthetic biologists work to optimize and improve biological systems. Experiments so far have shown promise. Light output has been low, but proofs of concept have been demonstrated and key areas for improvement highlighted. Fuelled by increased understanding of natural biochemistries and improvements in the technologies driving synthetic biology, engineered bioluminescence has a very exciting future.

2 Applications

Since the Promethean period more than 125,000 years ago when mankind began to nurture and control fire, this ability has set us apart from our closest relatives. The first light from flickering fires allowed social life to continue after darkness fell and became a key fixture of human civilization.

In the intervening millennia, technological advances have brought a succession of new approaches to lighting, from the grease lamps of antiquity, to candles, to the invention of the kerosene lamp in the Arab world. The nineteenth century saw the use of gas, then electricity, to emit brighter and cleaner light; the twentieth century brought the refinement of electric lighting to be more efficient through the use of fluorescent bulbs and light-emitting diodes (LEDs). Alongside this growth in our technology for producing light has come a range of new applications. The most obvious reason for artificial light is to illuminate dark spaces so that we may see (e.g. streetlights). But our uses go well beyond this simple use. Light has long been important for aesthetic purposes in culture and art, such as when awed crowds watch fireworks fly into the sky. We also use light to convey information to humans (traffic lights, television screens), and now even to convey information between machines (optic fibers). We will examine the contributions that synthetic biology may make to each of these areas in turn.

Light is a crucial component of many systems in conventional engineering, and there is no reason to suspect that this will change as we begin to engineer biological systems to tackle the challenges humans face. Thus, we can expect bioluminescence to play a key role in the continuing development of synthetic biology.

2.1 Illumination

The oldest application of light in the human world is the illumination of dark spaces. Paradoxically, this may be one of the last areas synthetic biology strays

into. The production of light from electricity by modern bulbs is efficient, and it is orders of magnitude brighter than any natural bioluminescent organism. This does not necessarily mean that synthetic biology will never rise to this challenge, as evolution has not selected for creatures to compete with light bulbs. However, such a competition is likely many years of biological engineering away.

Already there has been considerable discussion about the contributions synthetic biology may make to illumination. As part of their Microbial Home project, manufacturer Philips produced a concept design and prototype for a 'biolight'. The biolight consisted of tubes of blown glass containing bioluminescent bacteria, fed on methane gas from a biodigester processing household waste. The idea highlights one of the advantages of bioluminescent light over other approaches: it allows smaller-scale systems for the production of light from biomatter than any approach involving electricity as an intermediate.

Another favored concept has been the engineering of bioluminescent trees that use energy from photosynthesis to emit light at night. The first autoluminescent plants were created in 2010 [29], but they emitted only very dim light. A crowd funding campaign promising to distribute seeds for bioluminescent *Arabidopsis thaliana* recently raised almost half a million dollars [17] but also prompted considerable debate about the implications of such a release [8].

Due to the dim nature of current bioluminescent light, it seems likely that the first biolighting will come at the blurred boundary between aesthetics and illumination. Here, bioluminescence may be used not because of a practical advantage over other forms of lighting but because it allows the light to come from a more beautiful, living, light fixture.

2.2 Aesthetics

Bioluminescence in nature is a spectacle that has captured the imagination of artists and designers alike. In 2002, the Bioglyphs art/science collaboration [15] produced bioluminescent paintings inspired by nature plated out with *Aliivibrio fischeri*, and other artists have since conducted similar projects [14].

Natural bioluminescence has also started to be marketed towards consumers. Yonder Biology is currently taking preorders for the Dino Pet, a decorative light powered by photosynthesizing dinoflagellates [54]. However, without supplementation, the light lasts only for 1–3 months.

Synthetic bioluminescent approaches will allow the creation of lighting effects that would be difficult with electricity. Plankton or bacteria in suspensions create a glowing liquid that can be molded into shapes (as in the Dino Pet) or flow down transparent pipes, or perhaps be displayed with a fountain. Similarly, bioluminescent plants would emit light from a large surface area, creating a much more diffuse glow than bulbs that are almost point sources. We might imagine a path lit by an avenue of trees running down either side or bioluminescent Christmas trees that need no lights.

All of these ideas, however, require people to accept these genetically engineered organisms into their homes and environments. A precedent for genetically modified organisms living in households comes from the Glofish—genetically modified ornamental zebrafish expressing green fluorescent protein (GFP) so that they glow under an ultraviolet (UV) light. They are popular in the United States, where millions have been sold. There has been considerable debate about the potential ecological consequences should these fish be released into the environment, but some studies have been carried out to show that they are less fit than wild-type fish [23]. It is not difficult to imagine autoluminescent transgenic fish being developed to extend this idea. In another vein, the company Biolume has a large patent portfolio covering the use of bioluminescence in food and drinks, which it is working to commercialize [6].

2.3 Communication with Humans

Since the lighting of the flaming beacons that formed the first lighthouses, light has been an important medium for communicating information. It is now ubiquitous in this role, from the red, yellow, and green signals of traffic lights and crossing signs to the technicolor output of television screens and projectors.

Wherever a biosensor is used, whether to measure the levels of contamination in groundwater or the glucose in a diabetic person's blood, some mechanism is needed by which the molecular interactions that measure the target molecule can be turned into a human readable output. Today, this often involves an electronic intermediary, but here is the possibility that these systems might one day be entirely biological, with bioluminescent light replacing the status LEDs that exist today. Information could be conveyed by the simple presence or absence of light at certain spatial positions, or by output in colors that vary depending on the value to be communicated. The advantage of the latter approach is that it distinguishes a situation in which the biosensor is correctly reporting a negative result (true negative) from a situation where the biological material has been degraded and so is unable to emit light (false negative). For these applications, an intense light is needed at the point where the biosensor is read, but this may be a small area and the output may only need to last a short time.

Engineered luminescent organisms could serve as environmental biosentinels, surveying their ecosystems for possible dangers and pre-alerting us to unfavorable, damaging, or toxic conditions. More industrial applications are already being developed, such as bacteria to warn a company that its waste flow is too toxic and must be further treated or to report if groundwater is contaminated [52]. Synthetic biologists also hope that living bioreporters can bring applications to the consumer. Consider a garden plant glowing a particular color to remind you to wear sunscreen or take allergy medication.

Because luminescence is such an intriguing and beautiful phenomenon, it seems a fitting choice to consider engineered organisms with which we interact in this way—organisms that affect and inform us. It is also a safe reporter system, as the

proteins and chemical intermediates are non-toxic. The process is a burden on the host, so engineered bioluminescent organisms will be likely outcompeted in the environment; thus, the evolutionary advantages of bioluminescence are very niche, likely offering only negative selection pressure should the genetic system evade containment systems and combine into an unexpected host. Current-generation luminescent biosensors require detection with sensitive instruments. However, with improvements from synthetic biology, the results could be visible to the naked eye.

2.4 Communication with Other Engineered Systems

We now use light as a signal even where no human will see it. Computers communicate with optic fibers, with total internal reflection guiding the photons that carry data around our world. In a similar fashion, light can provide an ideal bridge within synthetic biology, both between biological and electronic systems and perhaps between multiple biological systems.

Bioluminescence is already a popular reporter in scientific research due to its ability to convey highly quantitative information. All popular biological chassis have very low background luminescence; the same cannot be said for other reporters such as fluorescence, which may have substantial and variable background levels. Light is easily detected by electronic components, including light-dependent resistors and photodiodes or, for lower levels, photomultipliers and avalanche photodiodes. Additionally, a single biological system can output data on a number of largely orthogonal channels by using luciferases that emit at different wavelengths. Two or more filters can be used prior to photodetectors in order to measure these channels independently.

The natural world provides a range of light-sensitive proteins which, when used in conjunction with luciferases, provide the potential for cell–cell communication using light. Proof of concept in a synthetic biology setting was carried out by the Peking iGEM team [31]. They used the previously developed light producing BioBrick to emit light in one culture of *Escherichia coli*. A separate culture, entirely separated by plastic, was then able to detect this light using a hybrid protein made by coupling a phototropin photosensor domain to a transcription factor and then express another protein—in this case, GFP. Bioluminescence can in theory allow communication between very different chassis—for example, between mammalian cells and bacteria—with light serving as a universal signal understandable by man, machine, or modified organisms.

Although these are some possible applications of bioluminescence in synthetic biology, new technologies can shift paradigms to such an extent that entirely new unexpected classes of applications become possible. It may be the case that advances in bioluminescence will one day be used in ways that we currently cannot imagine.

3 Feasibility of Bio-lighting

Most current bioluminescence applications in synthetic biology release light that is barely visible to the naked eye, requiring very sensitive electronic or chemical detection. It may seem ambitious, then, to propose that we might one day use the reaction for lighting our lives. Many projects have included back-of-the-envelope calculations on the feasibility of bioluminescent lighting, particularly whether or not a photosynthetic organism could derive enough energy from sunlight to give useful light output [43]. A detailed but by no means comprehensive analysis is given below, with particular focus on estimating whether a large luminescent tree could compete with a fluorescent streetlight.

3.1 Photosynthetic Efficiency

The sun delivers potentially vast amounts of energy. After some absorption from the atmosphere, approximately 1,321–1,471 W/m^2 of radiant energy falls on the earth at midday at sea level in North America [2].

Plants can only absorb in the visible region, and this photosynthetically active radiation (PAR) corresponds to roughly 45 % of total solar energy. Then, there are other losses, such as the reflectivity of leaves and the absorption spectrum of chlorophyll. The net result is that plants are able to theoretically use between 3 and 6 % of total solar radiation, corresponding to roughly 40–80 W/m^2 [43]. This energy is stored as chemical energy in biomass, although approximately half of the energy is usually lost through photorespiration or is used in respiration at night, leaving only around 1–3 % [21].

Photosynthetic efficiency—the fraction of light energy that plants can convert to chemical energy—has been thoroughly studied because of interest in energy crops for bioenergy. The efficiency varies greatly between plants based on their physiology and biochemistry. C_4 plants, for example, are typically more efficient because they avoid losses from photorespiration. Crop plants tend to have efficiencies of around 2 %; the C_4 plant sugar cane (*Saccharum sp.*) is more impressive, with typical efficiencies upwards of 6 %. The highest ever reported is a primrose in Death Valley, which claimed a photosynthetic efficiency of 8.5 % [34]. Fast-growing trees, such as willow and poplar, have photosynthetic efficiencies similar to crop plants [9]; approximately 2 % of the 1,400 W/m^2 is captured and stored, around 28 W/m^2.

3.2 Energy Output

There are several distinct measurements of brightness, including the following:

- Radiant flux, measured in watts
- Luminous flux, measured in lumens or candelas per steradian (cd sr^{-1}).

Radiance considers all electromagnetic radiation coming from an object, whereas luminance adjusts for the perceptive abilities of the human eye. A black object may have zero luminance but be giving out UV or infrared radiation and hence have high radiance. Luminosity functions (Fig. 1) show this weighting in the visible part of the spectrum; using these, one can convert from radiant flux to luminous flux. There are two curves shown: one for photopic (bright light, full-color) vision and the other for scotopic (dark) vision.

To convert from radiant flux to luminous flux, one must integrate the power spectrum weighted by the luminosity function so that wavelengths beyond that of human perception are cut out. Note that this transformation is therefore one-way. One cannot convert back from luminous to radiant flux. Due to the definition of the candela, an S.I. base unit, the maximum luminance for a light source is 683.002 lumens per watt of energy radiated. A 60-W incandescent light bulb does not emit 60×683 lumens, however. A great deal of energy is lost to heat and some of the radiant light is outside the perception of the human eye. This means that a typical incandescent bulb has a luminous efficacy of only around 15 lumens per watt.

From the emission wavelength of the chosen bioluminescence reaction and the luminosity functions for the human eye, one can calculate the luminance per watt for the particular reaction. The Cambridge iGEM team calculated this to be 471 lumens per watt for the *Aliivibrio fischeri* bioluminescence reaction observed with scotopic (low-light) vision [43]. Scotopic vision picks up blue/green wavelengths well, so only around one-third of the radiant energy is lost.

3.3 Competing with Conventional Lighting

Table 1 shows luminous outputs for typical street lamps. The values are in lumens and were adjusted using the scotopic luminosity function because streetlights operate in low-light conditions.

A light source created by a genetically engineered organism would require an output of at least 1,000 lumens to be at all competitive with conventional lighting. Let us assume that our tree absorbs all light passing through its projected area, which is a fair assumption because only a small percentage is able to make it through the canopy. Power stored from light around midday is around 28 W/m^2 of canopy. Over the 12-hour-day, however, the accessible light energy would only average around 14 W. We could engineer the tree to only output light in the dark, for say 8 h, giving it a theoretical maximum output (at 100 % efficiency) of 21 W/m^2. A large willow or poplar might have a canopy covering 30 m^2, giving it $21 \times 30 = 630$ W of power to use. This energy is calculated from photosynthetic efficiencies derived from measuring biomass production and hence gives the surplus energy available to a plant, after energy used to keep the plant alive. Wild-type plants might use this energy for growth or storage as starch. In engineered plants, we would hope to divert a portion of it to light production.

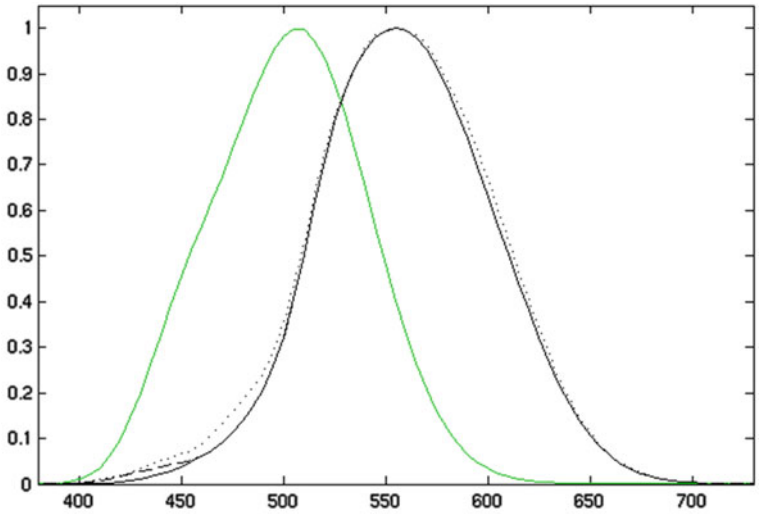

Fig. 1 Photopic (*black*) and scotopic (*green*) luminosity functions

Table 1 Approximate luminous output from typical streetlamps [43]

Light source	Output (lumens)
Incandescent	210–2,700
Fluorescent	1,000–7,500
Metal halide	1,900–30,000
High-pressure sodium	3,600–46,000
Low-pressure sodium	1,800–33,000

If the plant were to efficiently divert all of this spare energy to light output, then the tree would output $630 \times 471 = 296$ kilolumens. This is, of course, unfeasible as there will be energy losses in production or cycling of the substrates and because our engineering of living systems is far from perfect. However, we would only need to divert 0.3 % of this available energy to the emission of light to be somewhat competitive with conventional lighting. This is still challenging, but it may be achievable. Even with incomplete diversion of the resources and inefficient biochemistry, such a target may be within reach. The many other challenges, such as outer leaves blocking light from inner leaves, have possible solutions—in this case, perhaps shifting the emission wavelength a little more into the green part of the spectrum so it is minimally absorbed. It is not the physics holding back our bioluminescent future—only our abilities as scientists, for now. This is not to suggest that bioluminescence will ever replace conventional lighting. For high-output lighting applications, high-pressure sodium lights or LEDs will always be preferable. Bioluminescence instead has different qualities, being a softer, more diffuse light. It will likely find applications that complement traditional lighting.

4 Bioluminescent Parts and Modules in Nature

For a synthetic biologist, the natural world is an amazing catalog of parts and systems. The systems we find in nature may be altered, optimized, and transferred to new organisms or improved and edited in their natural host.

Throughout the history of life, bioluminescence has evolved many times. More than thirty independent biological light emission systems can be found in nature today, with potentially many more still undiscovered plus doubtless many sadly lost in evolutionary bottlenecks [20]. Among the diverse light-emitting organisms are bacteria, insects, fungi, and dinoflagellates. The luciferase enzymes, catalyzing the light-emitting reactions in these different organisms, show no homology to each other and their substrate luciferins are unrelated to each other chemically. The different reactions have different output wavelengths, efficiencies, and optimum conditions and the different substrates draw from different metabolic pathways for their production. For synthetic biology applications, we can choose to build from the most appropriate natural system for the intended chassis and end application. Our choices are limited, however, by biological understanding and sequence data for the natural systems.

The number of sequenced genomes is increasing exponentially, ever widening the potential parts list for synthetic biologists. Sequence information deposited in the National Center for Biotechnology Information Genbank database has continued to increase exponentially since its creation in 1982 (Fig. 2).

This increase is largely driven by the ever-decreasing cost of sequencing genetic material. Our abilities as synthetic biologists to edit and rewrite these genes are also increasing in a similar manner, driving the rise of the synthetic biology discipline. Carlson's curves, representing the observation that cost of sequencing and synthesis decreases exponentially, are shown in Fig. 3.

4.1 Firefly Luminescence

Of the known bioluminescent systems, firefly luciferases are particularly well studied and characterized due to their common use as reporter genes. The luciferase from *Photinus pyralis* was chosen for the first, now iconic, picture of a glowing transgenic plant (Fig. 4). The protein was expressed in tobacco plants from a cauliflower mosaic virus promoter, and the resultant GMOs were then watered with luciferin. Light emission was seen when plants were placed on photographic film, with the brightest light tracing out the water transporting vessels of the leaves and stem [38]. The reaction is known for its high quantum yield, emitting a strong yellow/green light [1]. This might make the system a good candidate for bio-lighting applications. However, despite the luciferase gene being sequenced in 1987, the genes for production of its substrate remain unknown. We cannot attempt to predict them, as the full firefly genome has not been sequenced.

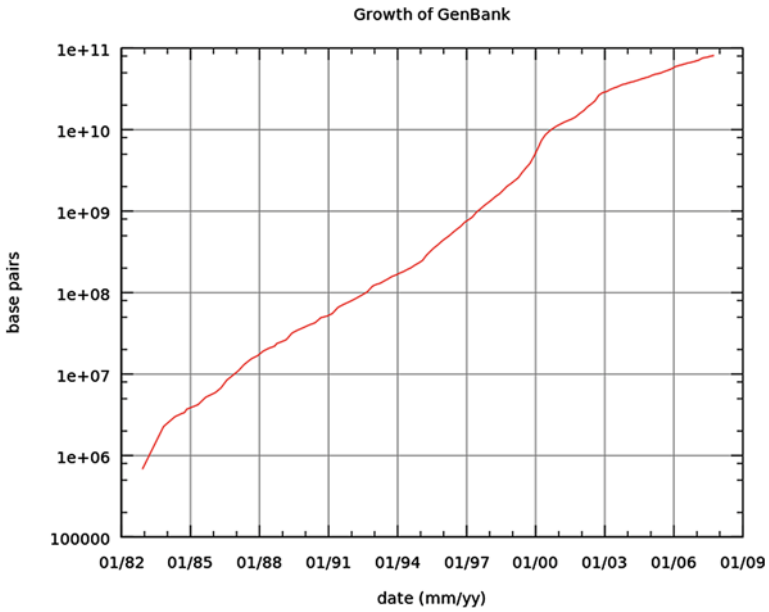

Fig. 2 Sequence information deposited in the Genbank database over time

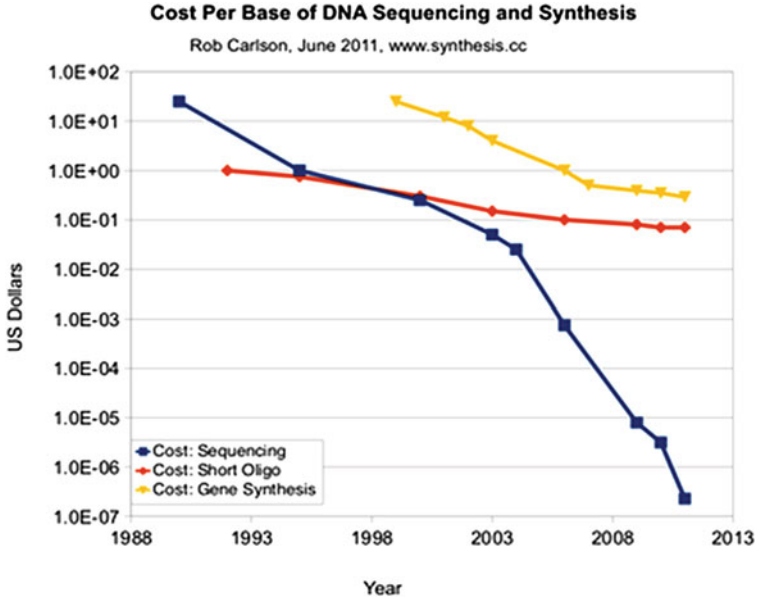

Fig. 3 The cost of DNA sequencing and synthesis over time. *Note* the log scale [10]

Fig. 4 An autoluminograph of a transgenic tobacco plant expressing firefly luciferase [38]. Copyrighted, Keith Wood (of the DeLuca lab) and Science magazine

With sequencing becoming ever cheaper and our abilities to annotate genomes and characterize parts improving, this will change. For now, however, the reaction in transgenic organisms requires the addition of expensive luciferin substrate.

The presence of an enzyme for recycling the luciferin substrate was suggested in 1974 [37]. The researchers discovered that radioactively labelled oxyluciferin and 2-cyano-6-hydroxybenzothiazole would be converted into luciferin when injected into living fireflies. Nearly 30 years later, the protein involved was identified and named *luciferin-regenerating enzyme*, or LRE [19]. This enzyme was shown to recover D-luciferin with the addition of D-cysteine. Oxyluciferin can block the firefly luciferase active site, so it is a competitive inhibitor of luminescence; its removal by LRE improves light output in vivo, with the addition of D-cysteine prolonging output even further [43]. This is a step towards sustained light output in a modified organism from the firefly system. Genes for de novo luciferin biosynthesis would only have to produce a little of this substrate, and it could then be recycled near indefinitely via the LRE cycle. Most organisms do not produce D-cysteine, however, as it is acutely toxic to mammals and a strong inhibitor of bacterial growth. Therefore, it may be difficult to establish this LRE cycle in

alternative organisms. Possible recycling via L-cysteine has also been reported [36]; although it is not yet shown in synthetic organisms, in the future this may be the pathway of choice for recycling luciferin in bio-lighting (Fig. 5).

A different reason for employing the firefly system in bio-lighting is the broad spectrum of colors that are possible. It was noted that firefly luciferases in vitro were pH sensitive, and their color could vary from yellow/green to red depending on the conditions. In vivo, a range of colors is also possible; mutants have been produced, which show different spectral outputs at the same physiological pH [24]. Single amino acid changes were introduced in the Japanese firefly (*Luciola cruciata*) luciferase, another popular reporter protein. These substitutions change the peak wavelength of light output, visibly altering the color luminescence (Fig. 6).

4.2 Bacterial Bioluminescence

Aside from fireflies, luminous bacteria have also received considerable attention. Luminescent species are all gram-negative, non-spore-forming, motile bacteria; they are the most abundant and widely distributed of all light-emitting organisms. They can be found as free-living species in the ocean, as saprotrophs on dead marine organisms, as symbionts in the light organs of fish and squid, and in many other ecological niches. Known luminescent bacteria are found in the genera *Aliivibrio*, *Photobacterium*, *Alteromonas*, and *Photorhabdus*. The bacterial enzymatic light-emission system, encoded by the lux operon, is highly conserved amongst the various species of luminous bacteria, with the most common architecture of the operon represented by *luxCDABEG*. The bacterial luciferase uses flavin mononucleotide and a long-chain aldehyde, derived from fatty lipid biosynthesis as substrates for the light-emission reaction. The *luxA* and *luxB* genes encode α and β subunits of the bacterial luciferase; *luxC*, *luxD*, and *luxE* encode enzymes involved in the synthesis of aldehyde substrate; and *luxG* codes for flavin reductase, which participates in flavin mononucleotide turnover [29]. This inbuilt natural modularity is useful for synthetic biology. The operon contains all the genes necessary for bioluminescence, production, and cycling of the substrate plus the light-producing reaction all together. This makes its study and transfer to certain new hosts considerably easier. It may also be a limitation, however—eukaryotic nuclear DNA does not contain operons and the cytosolic ribosomes cannot usually process them, so the genes would require considerable refactoring for these hosts.

Bioluminescent *Escherichia coli* were produced in the 1980s with the operons from *Vibrio harveyi* and *Aliivibrio ficheri* [33]. Early experiments with *Agrobacterium*-mediated transformation involved only the luciferase enzyme. *Vibrio harveyi luxA* and *B* were expressed in transgenic tobacco and carrot cells and shown to correctly assemble into functional luciferase with light produced from extracts with substrates added [28].

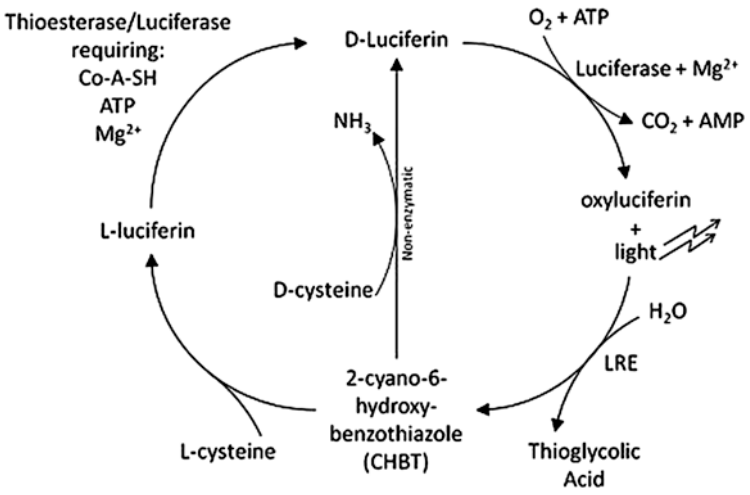

Fig. 5 Possible luciferin-regenerating enzyme cycles [43]

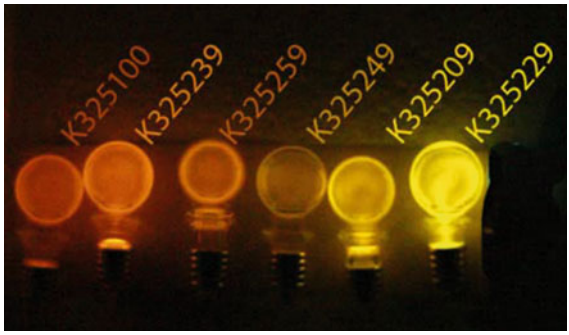

Fig. 6 Five different mutant firefly luciferases and the wild-type (*second from right*) expressed in *E. coli* with luciferin added to the media [43]

Transgenic autoluminescence in a higher eukaryote was only achieved decades later with the refinement of ballistic transformation methods. The entire *Photobacterium leiognathi lux* operon was transformed into the chloroplasts of tobacco plants and produced luminescence [29], discussed in detail later). In the same year, the whole operon—with genes chosen from *Photorhabdus luminescens* and *V. harveyi*—was expressed in mammalian cells but required considerable refactoring. Codon usage was reoptimized and the six genes were split into three bicistronic pairs, with a viral internal ribosome entry site (IRES) between the pair. This allowed weakly autoluminescent *HEK293* cells (a human cell line) to be produced.

Other attempts at bio-lighting have used the *V. ficheri lux* operon. When expressed from an arabinose inducible promoter in *E. coli* and activated by the addition of L-arabinose, large flasks produced enough light to read by [43] (Fig. 7).

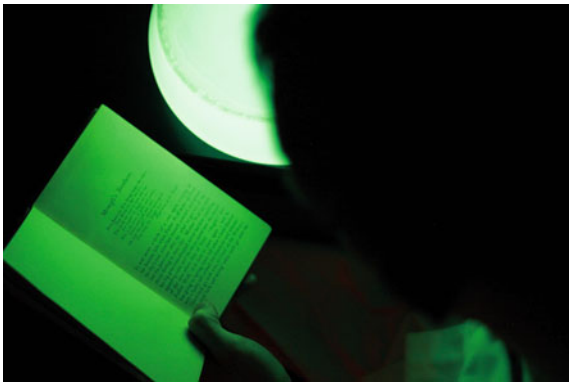

Fig. 7 *The Jungle Book* read by transgenic bioluminescence [43]

4.3 Other Natural Luminescence Systems

Fungal bioluminescence, colloquially known as "foxfire," is an impressive sight in temperate or tropical woodlands (Fig. 8). More than 70 terrestrial bioluminescent fungal species have been described; however, their biochemistry is relatively poorly understood. Possible luciferins have been found, but the pathways for their production remain unknown. No luciferase enzymes have been sequenced; indeed, the existence of a luciferase at all was only recently confirmed [45]. Previous theories suggested that light was produced by an uncatalyzed reaction between metabolites. Unfortunately, this lack of information is a barrier for consideration in synthetic biology applications. There may be wonderful luminescent genes and systems hidden in fungi, but the genetic and biochemical understanding must catch up to that of insects and bacteria before they can be used.

There are similar knowledge barriers for the systems from numerous other luminescent organisms. Considered species include dinoflagellates, which are responsible for luminescent waves sometimes seen in tropical waters; cnidaria (the phylum containing jellyfish and anemones), such as the sea pansy *Renilla reniformis;* and copepod crustaceans, such as *Gaussia princeps*. For these organisms, the luciferase enzymes and their substrates—a chlorophyll derivative in dinoflagellates and coelenterazine in cnidarians and copepods—are well known, but the rest of their biochemistry is not. The lack of described biochemical pathways for substrate production and knowledge of the genes involved prevents experimentation in synthetic biology applications for now. However, this will change as our understanding of the natural world grows. As our curiosity to learn about living systems in nature drives new discoveries, it also equips us with the parts and knowledge to design and build new living systems of our own.

Fig. 8 Bioluminescence in the fungus *Panellus stipticus* shown in a long-exposure (~8 min) *photograph*

5 Selecting Chassis Organisms

To differentiate synthetic biology from genetic modification (GM), a comparison to computing is often made. If GM is tweaking the operating system, then synthetic biologists attempt to develop applications (apps)—new genetic programs that operate somewhat orthogonally from the host system and confer a particular new function. Because of the universal genetic code and central dogma common to all life, we might hope each app will function in a variety of organisms, but they inevitably interact differently with each host. Well-characterized host organisms (chassis) make these interactions more predictable, but our choice may be limited due to the application requiring a chassis with particular features or adaptations to particular environments.

E. coli is the workhorse of microbiology research and the most popular synthetic biology chassis with the best characterized genetic parts available to it. As a gut commensal, most strains have been generally recognized as safe; attenuated laboratory strains are unlikely to survive in the environment. This makes *E. coli* without a doubt the chassis of choice for study in the laboratory; it may also find real-world bio-lighting applications. Household lighting systems of engineered luminescent bacteria fuelled by household waste have been proposed [39, 43] and design prototypes were produced as part of Philips' design futures projects (Fig. 9). Like all bio-lighting, the intention is to complement rather than replace conventional lighting. In this case, it might offer a sustainable alternative for atmospheric interior lighting.

The prospect of moving bioluminescence into plants has particularly captured the imagination of scientists, the media, and the public. Practical proposals, such as the replacement of streetlamps with luminescent trees, or more aesthetic and artistic applications, such as self-illuminating Christmas trees or houseplant mood lighting, are met with wonder and excitement [22, 47].

Progress is certainly being made towards these futuristic proposals. The iconic tobacco plant luminograph (Fig. 4) was the first experimental demonstration of bioluminescence in plants, requiring the addition of the luciferin substrate [38]. It

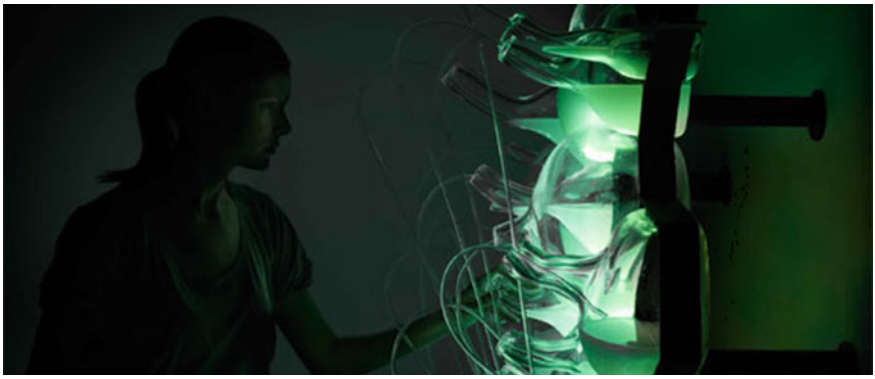

Fig. 9 A design idea for luminescent bacteria feeding off household waste [39]

took over two decades for the first autoluminous plants to be created [29]. There is now renewed interest in the area, particularly amongst students and in community science projects [5, 43].

Krichevsky et al. were able to engineer a complete functional bacterial luciferase pathway in tobacco plastids to produce both luciferase and substrate luciferins. Remarkable in its proof of concept for future bio-lighting, the experiment also showed the first complete and functional foreign biochemical multienzyme pathways to be engineered in plastids. Amongst the different luminescent systems, the bacterial light emission system was chosen due to the cyanobacterial evolutionary origins of plant plastids. The evolutionary similarity with plastids means that both plants and bacteria similarly manufacture riboflavin, from which the substrate flavin mononucleotide is produced. The fatty acid biosynthesis pathway, for production of the aldehyde substrate, is supported by the same type II fatty acid synthase in both plants and bacteria [44]; this is in contrast to animals and fungi, where fatty acids biosynthesis is mediated by type I fatty acid synthase.

Plastid translation machinery also allows expression of multigene operons and lacks nuclear transgene silencing mechanisms, which might be detrimental for nuclear expression of the complex multigene pathway. The relatively small ($\sim$155 kb) plastid genome in tobacco is present in thousands of copies per cell, allowing many copies and higher expression of the transgenes.

The plastid host operating system may seem like the perfect place for the bacterially derived bioluminescence app, but there will always be incompatibilities. These organelles are not bacteria and fundamental biological differences exist. For example, many of the chloroplast-encoded open reading frames do not have Shine-Dalgarno sequences, which are required for bacterial translational initiation; in addition, chloroplast initiation codons are not limited to AUG or GUG like free-living eubacteria. Other differences occur in promoter architecture, posttranscriptional RNA processing, and protein folding. Differences like these will always present challenges for synthetic biologists to overcome or work around.

Krichevesky et al. selected a strong plastid promoter and cloned the whole *Photobacterium leiognathi luxCDABEG* operon, unmodified, upstream of it. Two candidate integration sites were selected and vectors were produced, with the operon flanked by suitable regions for homologous recombination. Plants were transformed by particle bombardment. Amazingly, for the integration into the more transcriptionally active loci, autoluminescencent plants visible to the naked eye were produced. It was rather fortunate that the *P. leiognathi* operon structure and ribosome binding sites function in *Nicotiana tabacum* plastids and that the RNA and proteins were stable, with only the promoter needing to be changed. The light output was promising but rather weak; whole plants were visible to the eye only after 5–10 min of dark adaption or with a consumer camera and a 5-min exposure time (Fig. 10). Shoots placed in scintillation counter vials emitted around 1.4 million detected photons per second—half a mere picoWatt (10^{-12} W) in power. The luminescent plants had no phenotypic differences or altered growth compared to wild types; the light output was clearly a negligible energy expenditure. There are many obvious areas for improvement. The bioluminescent genes had not been optimized for the plant system and we could engineer differences in the host metabolism to better support the foreign bioluminescent pathway.

The experiments in tobacco laid the foundations for moving luminescence into other plants. Plastid transformation seems to be a promising option for other future plant chassis. In addition to the higher possible gene copy number, chloroplasts are generally restricted to the leaves, so energy is not wasted on hidden glowing roots. In only being maternally inherited, plastids are not present in pollen, which allows a level of containment of the foreign DNA.

Work in the primary model plant *Arabidopsis thaliana* is underway [17] following similar procedures for integration of a bacterial *lux* operon into chloroplasts or by *Agrobacterium*-mediated nuclear transformation. Trials and improvements for light output from fast-growing model plants could inform plans for future scaling up into larger organisms. A plant-optimized bioluminescence system developed in *Arabidopsis* could be relatively easily installed in a fellow eudicot/rosid plant, such as poplar or willow.

For larger-scale bio-lighting applications, a large woody plant chassis would be best suited and a well-researched, fast growing tree species would be ideal. *Populus trichocarpa* was the first woody plant to have its genome sequenced, published in 2006, and it can be reliably transformed. Driven by its cultivation for timber, pulp, and paper plus increasing consideration as a possible energy crop, poplar physiology and biochemistry are well studied, and it has arguably become the model tree [49]. Alternatively, willow is also fast growing and can be grown on poor-quality land requiring minimal fertilizer. Particularly hardy strains exist that are tolerant to flooding and drought [25]. The purple willow *Salix purpurea* genome has been mapped and sequencing is underway. Willow cannot be transformed by *Agrobacterium,* but ballistic transformation methods are under development and look promising [16]. Plants also engineered to be hardy and tolerant to harsh environments could be used to provide cheap lighting in off-grid areas where traditional street lighting might not be economically or logistically possible.

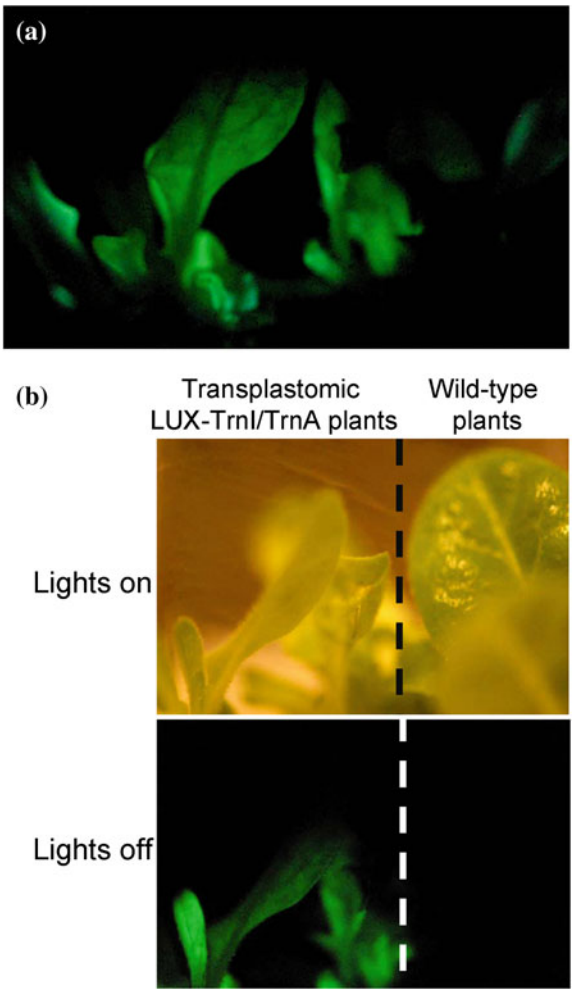

Fig. 10 Autoluminescent tobacco seedlings [29]

For more aesthetic bio-lighting projects, luminescent ornamental species have been suggested. Gardening and flower arranging could benefit from the surreal beauty of bioluminescence. The rose (*Rosa hybrida*) is a prime candidate [17]; the genome has been mapped, and it can be transformed by agrobacterium and ballistic methods. The prospect of doing away with fairy lights at Christmas in favor of an autoluminescent tree is also appealing. Studied because of their importance to the forestry industry, pine and spruce trees have also been transformed in attempts to improve tolerance to drought and other stresses by genetic engineering [48]. The most popular Christmas tree species in Europe, the Norwegian spruce (*Picea abies*), can be transformed with high efficiency and has a draft genome completed. The traditional North American Christmas tree, the Douglas fir (*Pseudotsuga menziesii*), has a draft genome in progress.

Biological understanding, sequence information, and transformation methods are the necessary features for synthetic biology chassis, so higher plants offer many options for ambitious future synthetic biologists (Fig. 11).

6 Controlling Luminescence

Synthetic biology attempts to make biological systems more reliable, predictable, and controllable. Naturally, any biosensor application requires that luminescence is tightly controlled based on an input. For lighting applications, we might only want luminescence at a particular time of day, or we might want our organisms to report on their environment—for example, glowing in response to pollution or offering a reminder to wear sunscreen when the UV index is high. Through regulating the luminescence gene networks, engineers could also create a multitude of programmable colors and patterns.

Luminescent reporters as used in current research generally modulate light output by changing the level of transcription of the luciferase gene. This creates a model that is slow to react to changes because the protein's half-life is on the order of hours. Some approaches have destabilized the protein in order to give more responsive regulation [30], but this approach still has limits. Nature shows us that very tight control of luminescence is possible, with Japanese fireflies able to synchronize their flashes to within milliseconds. This is made possible by the fact that the fireflies' luciferase is localized to the peroxisomes. Nerve activity, relayed by nitric oxide signaling, causes adenosine triphosphate either to be consumed by the mitochondria or allowed to enter the peroxisomes to produce light. These sorts of approaches may be necessary to achieve luminescent light that is activated instantaneously as if by a light switch.

For lighting applications, a chassis would have limited resources to devote to luminescence. Brightness could be increased by restricting light output to certain tissues or to certain times of day. The clock genes in Arabidopsis are a model for circadian rhythms and are well characterized [41]. Glowing plants would only be visible at night anyway, so it would be sensible to have luminescence genes controlled by a clock gene promoter. Plants also track the seasons—perhaps flowering in spring and setting seed in autumn. They are able to achieve this by using photoreceptors to monitor the changing day length. By tapping into the gene networks that are modulated as a result, a gardener might be able to cultivate plants that only glow when they flower or that are specifically designed to brighten up the dark winter months.

A common dream of future-gazing synthetic biologists is a natural world that talks to us, displaying warnings of pollution or operating as natural clocks. Biosensors are a key application area for current synthetic biology and an area that will only grow with our increasing knowledge. The modular nature of synthetic biology means that any detector module could be connected to any bioluminescent module to allow different light outputs to communicate changes occurring at a molecular level in a way that is easily visible and aesthetically pleasing.

Fig. 11 Might luminescent trees light our future cities? [43]

7 Containment

Many synthetic biology applications of bioluminescence would involve deliberate release of the modified organism into the environment. This requires safety to be seriously considered because the organisms or modified genes could have long-term effects on the environment.

Today's concerns revisit questions raised previously during the introduction of recombinant DNA technology. The 1975 Asilomar conference was the most influential discussion forum for such issues; here, a cautious approach was suggested with both physical and biological containment recommended to minimize the environmental risks that GMOs may pose [4]. Decades later, these principles have ensured no significant disasters [3]. In 1982, the Organization for Economic Co-operation and Development produced a report considering the potential hazards of releasing GMOs into the environment, with particular consideration given to transgenic plants. The same cautious approach to the science and its regulation was suggested, including trials to assess ecosystem impacts [7].

In response to the continued advances in synthetic biology, an influential review also reaffirmed these principles and decided they should extend to the use of novel sequences not found in nature [42]. It also recommended that synthetic biologists constantly consider new risks and impacts in an ongoing process of prudent vigilance.

The success of the Asilomar approach so far may be due to careful regulation, but also to the limits of subsequent environmental GMO releases. Few engineered microbes, which are more prone to horizontal gene transfer, have been released.

Those that were released have been laboratory-acclimatized cells, largely unable to establish themselves in the environment [11]. Genetic alterations in released higher organisms have so far been relatively minimal, adding single genes or tweaking expression. Synthetic biology promises more complex, larger-scale modifications [26], which potentially bring more significant or unpredictable impacts to ecosystems.

We must work to consider and minimize these risks on a case-by-case basis. For bio-lighting (and indeed with all GMO releases), there are two areas of concern. Firstly, could the GMO outcompete native species or otherwise disrupt habitats? Secondly, could altered or synthetic genetic material escape its host and contaminate indigenous organisms?

Bioluminescence is inherently very safe: proteins and chemical intermediates are nontoxic and the process, particularly if optimized for bio-lighting, places a huge burden on the host; therefore, engineered bioluminescent organisms will likely be quickly outcompeted in the environment. Even so, the light may have effects on other organisms if the GMOs escaped. For example, moths and birds are confused at night by artificial lights in cities [32] and would suffer more if brightly glowing plants were to overpopulate rural areas. To minimize the release of plants, we might consider only distributing sterile seedlings by introducing mutations preventing flowering, killing pollen, or preventing viable seed setting. We might also contain the plants with auxotrophy—engineering a dependency upon an externally provided molecule for growth. Many molecules have been demonstrated including methionine, biotin, and auxin. These allow normal propagation of the plants when watered with the required nutrient but prevent growth should they escape. For microbes, we might also employ auxotrophy in addition to using attenuated laboratory strains that are easily outcompeted by wild microorganisms. Cells could also be physically contained within an alginate gel, for example (Fig. 12A) [51].

The evolutionary advantages of bioluminescence are niche, so the genetic system is unlikely to be under any positive selection pressure should it evade genetic containment systems and combine into an unexpected host. However, fitness effects of transgenes may be somewhat unpredictable [50] and interactions of unknown hosts within the ecosystem even more so. Therefore, genetic containment must always be seriously considered.

In addition to minimizing gene transfer, we should also consider DNA barcodes to trace synthetic biology designs. With DNA sequencing becoming more rapid and affordable, direct sequencing of environmental samples can now be used to identify contaminating synthetic DNA [51]. Designs can contain genetic 'barcodes' that could be used to identify their origin. DNA watermarks have been inserted in multiple genomic locations to aid in identifying engineered cells [18]. Barcodes not only aid in identifying genes in the environment, but could also be used commercially to mark and track proprietary strains.

For multicellular organisms such as plants, horizontal gene transfer is rare; therefore, genetic containment can go hand in hand with physical containment. Growth can be restricted to particular areas with auxotrophy and viable pollen

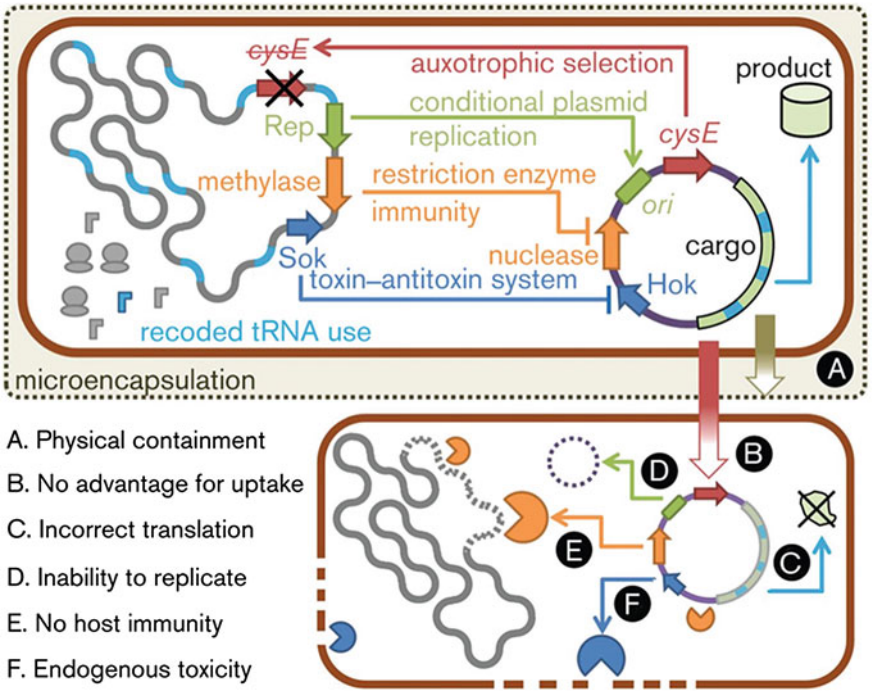

Fig. 12 Genetic containment mechanisms [51]

formation can be prevented. Microorganisms are prone to horizontal gene transfer; hence, steps should be taken to reduce the chance of the synthetic DNA finding unintended hosts. Many such mechanisms exist, particularly if the synthetic construct is a plasmid; this allows systems where the plasmid is dependent on the intended host, and vice versa (Fig. 12).

The strongest 'genetic firewalls' have synthetic DNA cargo that contains sequences that can only be correctly translated by engineered bacterial hosts with refactored transcription or translation machinery (Fig. 12C). Although still under development, such mechanisms represent the most failsafe containment options for the future.

Such orthogonality is possible in a number of ways. One approach involves refactoring a cell's codon usage such that it no longer follows the universal genetic code. Certain codons and their associated tRNAs may be changed such that the intentional cell host cell inserts different or even unnatural amino acids for particular codons [12]. Translation of an ltered synthetic gene from such a system by an unintended natural host natural organism would give a nonfunctional, mistranslated product. Alternatively, evolved ribosomes that recognize nonnatural ribosome-binding sites for translation or translate recognizing a quadruple-base-pair code are another way of obtaining this genetic code orthogonality [35].

Both of these approaches use natural nucleic acids. Synthetic variants may also find applications. Alternative base-pair combinations functional with natural DNA polymerases in vitro have been found [53], and research to show these synthetic bases work with plasmids in vivo is ongoing. Xeno nucleic acids (XNA), where the backbone sugars of DNA are changed, are not recognized natural DNA and RNA polymerases, but polymerase mutants that can use DNA as a template for XNA synthesis and vice versa have been developed [40]. The use of XNA in vivo is many years away, but it hints at the most fail-safe separation of our engineered constructs or organisms from the natural world.

With no single perfect containment mechanism, the current consensus is that deployed GMOs should use multiple containment methods. This redundancy would present a GMO or genetic construct with several evolutionary hurdles to overcome simultaneously in order to escape, therefore greatly safeguarding against 'life finding a way' [51]. We must be careful, however, to be proportionate and to avoid stifling innovation [42]. Containment must match the risks. Field trials of bioluminescent trees for lighting may show no danger of growth in the wild, negating the need for auxotrophy, although we might recommend male sterility. Careful case-by-case, evidence-based risk assessments are vital to safely pursuing a bioluminescent future.

8 Improving and Altering Luminescence

Synthetic biology draws much inspiration from engineering; optimization is one such concept. By studying, characterizing, and testing systems, we can redesign and improve them. Current bioluminescent light, in nature and especially in the laboratory, is very dim. However, a number of approaches may allow light output to be increased, thus allowing new applications.

There are a number of simple changes that may be made to increase the levels of proteins for bioluminescence, such as selecting stronger promoters or better ribosome binding sites. If constructs are being synthesized de novo, luminescence genes can be codon-optimized for expression in the target chassis. For example, the *lux* operon from *V. fischeri* has now been optimized for expression in mammalian cells [13]. In cases such as this, where an operon is transcribed from a single promoter in a bacterium, eukaryotic expression requires either inserting an additional promoter between each gene or inserting ribosomal skipping sequences to ensure each protein is processed separately.

To optimize luminescence, it is important that the products of light emission are recycled as efficiently as possible to new substrate for further reactions. Approaches to achieve this include the expression of luciferin-regenerating enzyme for firefly luciferase or the *luxG* flavin reductase in the *lux* operon. Further optimization will involve analyzing the quantities of metabolites at each step of the luminescence reaction. The rate-limiting step can then be identified. It might be the amount of luciferase or luciferin present, or of some intermediate. The ratio of

enzyme abundances could be tweaked in an attempt to remove this bottleneck and the process iterated. With each iteration, the metabolic processes culminating in light output should become more efficient.

An alternative approach is to allow nature to make some of these alterations itself through the process of directed evolution. An initial light emission system could be expressed in bacteria; then, those bacteria could be mutagenized. Optical selection of the brightest colonies, and then iteration of the process, should eventually produce brighter light-emitting modules. The advantage of this approach is that it is not limited by our own understanding of what mutations might increase light output.

As well as increasing light output, changes to the sequences of luminescent proteins may make them more tolerant of a range of pHs or temperatures [27]. Such flexibility would be especially important when proteins were expressed in heterologous systems, which may be quite different to their natural host. This is especially important for bioluminescent systems from sea-living creatures that typically function poorly at the warmer temperatures common in some chassis (e.g., 37 °C).

The microenvironments in which bioluminescence occurs may also be important for improving the light-emitting reactions. Looking to nature for inspiration, we see that some species of dinoflagellate have specialized organelles, the scintillons, which are optimized for their bioluminescent reactions. Ultimately, synthetic biologists hope to be able to realize such structures, and this may again help to optimize luminescence.

8.1 Alterations

There are a number of ways in which synthetic biologists might alter the spectrum of wavelengths, and thus the color, of light emitted in bioluminescence reactions. Subtle changes in the amino acid sequence of luciferase enzymes can be enough to significantly alter the color they emit (e.g., from green to red in firefly luciferase; [24]. An alternative to changing the sequence can be simply to change the pH at which the reaction occurs, which can have marked effects on luminescence.

Another approach is to change the chemical structure of the substrate for the enzyme, D-luciferin, in the case of firefly luciferase. So far, this has been achieved by chemists rather than synthetic biologists, given our ignorance of the biological pathways that produce D-luciferin, but it has allowed the production of a wide variety of color-giving luciferins [46]. Once we better understand the metabolic processes by which luciferin is produced, it ought to be possible to replicate these results with variant enzymatic pathways.

Finally, a more low-tech solution to altering the color of light emitted by a luciferase is simply to change the light's color after it has been emitted. This can be achieved using fluorescent proteins, provided the desired wavelength is longer than that already being emitted. There are many natural and engineered fluorescent proteins available, with a broad range of excitation and emission frequencies.

9 Conclusions

Natural bioluminescence is wondrous, efficient, and likely to be a key part of biological engineering in the future. Exciting proposals range from the strictly practical to the deliberately beautiful. Bioluminescence is potentially feasible as a source of useful illumination, although it will never exceed conventional lighting in terms of sheer brightness. Instead, it seems likely to complement existing light sources, offering a more aesthetic alternative or conveying useful information.

However, there are many challenges along the way. Our knowledge of natural bioluminescent systems and of synthetic biology strategies to transfer and optimize them is limited but ever increasing. Of course, we must be responsible with the design and implementation of new technologies and consider their wider impacts. Care must be taken to identify and minimize any risks. A synthetic biology approach values modularity and good characterization of parts so that adaptable bioluminescent systems can be produced and optimized for a range of applications.

Luminescent biosensing and communication applications are still in their infancy and bio-lighting is only theoretical at this stage, but many studies have proven the principles and highlighted the areas of improvement needed. With knowledge and techniques that are ever improving, increasingly amazing bioluminescent systems and organisms will be available to scientists and consumers. These developments could herald a bright and exciting future.

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Optimization of the Firefly Luciferase Reaction for Analytical Purposes

Arne Lundin

Abstract The optimization of assays has two purposes: (1) to increase the sensitivity of the assay so that low levels of the analyte can be determined; and (2) to prevent small changes of the reaction conditions from having a large impact on the outcome of the assay. The two purposes are usually equally important, as has been recognized in well-established branches of analytical chemistry, such as clinical chemistry. The firefly luciferase reaction can be used for many types of assays. The way to optimize these assays is not trivial, as there are many parameters to consider. Furthermore, as there are now several types of recombinant luciferases available, one has to decide which is the most suitable for each individual assay. The optimization is influenced by the conditions and requirements under which the assay is performed. Special attention is given to ways to calibrate assays. Examples on optimization are mainly taken from the author's own work during 40 years using assays based on the firefly luciferase reaction.

Keywords Assay · ATP · Bacteria · Bioluminescence · Cell · Detection · Firefly · Hygiene · Luciferase · Luminometry · Optimization

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
amol	Attomole(s)
DAPP	Diadenosinepentaphosphate
EDTA	Ethylenediaminetetraacetic acid
mol	Mole(s)
μmol	Micromole(s)
nmol	Nanomole(s)

A. Lundin (✉)
BioThema AB, Handens Stationsväg 17, 136 40 Handen, Sweden
e-mail: arne.lundin@biothema.com

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Contents

1 Introduction 32
2 Pretreatment of Samples 34
2.1 Concentration of Bacteria 34
2.2 Dilution 34
2.3 Pretreatment to Remove Specific Pools of ATP 35
2.4 Resuscitation 36
3 Extraction of Intracellular ATP 36
4 Selection of Luciferase for Different Applications 37
4.1 Reporter Gene Assays 38
4.2 ATP Assays 38
5 Luciferin 39
5.1 D-Luciferin 39
5.2 Luciferin Analogues and Derivatives 40
6 Kinetics of the Luciferase Reaction 40
7 Optimization of Reagents 44
7.1 Optimization of Assays of ATP 44
7.2 Optimization of Assays of Luciferase 45
7.3 Optimization of Coupled Enzyme Assays 46
8 Stabilization of Reagents 52
9 Calibration of Assays 52
10 Sources of Background and Ways to Reduce Them 56
11 Effect of Measuring Different Light Parameters/Read-Outs 58
12 Conclusions 58
References 59

1 Introduction

Firefly luciferase catalyzes the reaction:

$$\begin{aligned} &\mathrm{ATP} + \text{luciferin} + \mathrm{O_2} \overset{\text{luciferase}}{\longrightarrow} \\ &\mathrm{AMP} + \mathrm{PP}_i + \text{oxyluciferin} + \mathrm{CO_2} + \text{light} \end{aligned} \tag{1}$$

The reaction can be used for measuring luciferase, adenosine triphosphate (ATP), D-luciferin, or oxygen. The first two purposes are the most frequently used. With respect to ATP and D-luciferin, the reaction essentially follows the Michaelis–Menten equation:

$$v/V = S/(S + \mathrm{Km}) \tag{2}$$

where v is the rate of the reaction in the presence of substrate concentration S, V is the maximum reaction rate when $S \gg \mathrm{Km}$, and Km is the Michaelis–Menten constant (i.e. the substrate concentration resulting in a half-maximal reaction rate). When measuring luciferase, the substrate concentrations will be high to give maximum light emission. When measuring ATP, one has a choice depending on

the required sensitivity. At low luciferase concentrations, ATP is only slowly depleted and the light emission is almost constant provided luciferase is not inactivated in some way [1]. This is convenient when monitoring ATP-depleting or ATP-forming reactions. At high luciferase concentrations, ATP is depleted in a first-order reaction:

$$S = S_0 e^{-kt} \tag{3}$$

where S_0 is the initial ATP concentration, k is the rate constant, and t is the time. This gives a high initial light emission, which rapidly decreases as ATP is depleted. Under these conditions, a high sensitivity and a low detection limit for ATP is obtained. It is actually possible to detect 1 attomole of ATP, or the amount in a single bacterial cell (Berthold Detection Systems Application Note 2010/01).

Much of the basic work on the enzymology of firefly luciferase came from the group of McElroy and his wife DeLuca, including that the reaction required ATP [2], the structure and synthesis of D-luciferin [3], purification of luciferase to crystalline state [4], and recombinant production of luciferase [5]. The energy for forming a photon does not come from ATP (as McElroy thought in 1947) but from the oxidative decarboxylation of D-luciferin. The spectral emission and quantum yield has been described by Seliger and McElroy [6].

It was soon realized that the luciferase reaction had a great potential as a powerful analytical tool in many areas [7]. Optimized and standardized ATP reagents did not become available on the market until 1978 at the first International Symposium on Bioluminescence and Chemiluminescence. The reagent was based on the concept of continuous monitoring of ATP-converting reactions by measuring the light emission [8]. An early application in clinical bacteriology was bacteriuria detection in the diagnosis of urinary tract infection [9]. The first application in clinical chemistry was creatine kinase isoenzymes (CK) [10–14]. The major application has, however, been in the bacteriological control of food and beverages. In these industries, ATP has also been widely used for monitoring surface hygiene. This application is now spreading into hospital hygiene.

There are three major reasons why the firefly luciferase reaction has become so widely used for analytical purposes. Firstly, light can be easily detected at very low levels with simple instrumentation providing a high sensitivity. Secondly, light emission can be measured in different types of vessels, such as cuvettes, microplates, Petri dishes, on surfaces, and even in living animals or plants. Thirdly, ATP is the energy currency in all living cells being acted upon by hundreds of enzymes. Throughout evolution, cells have adapted the Km values of most of their enzymes to be able to regulate the enzyme activities. Consequently, the intracellular ATP concentration is similar in most living cells. The amount of ATP per cell is therefore mainly determined by the intracellular volume; a normal bacterial cell contains 1–2 attomoles ATP, whereas the larger mammalian cells typically contains 10,000–100,000 attomoles.

This chapter describes which parameters to consider when optimizing assays based on the firefly reaction. Most examples were taken from the author's own

work during the last 40 years—not because these are necessarily the best assays ever developed, but at least the motives for the way to optimize are known to the author.

2 Pretreatment of Samples

Samples often have to be pretreated to allow the assay to be performed. The pretreatment can be a concentration to provide the sensitivity required, for example, but it can just as often be a dilution to stay within the linear range of the assay.

2.1 Concentration of Bacteria

Although the amount of ATP in a single bacterial cell can be detected with supersensitive ATP reagents, special treatments to remove ATP contamination from pipette tips, cuvettes, or microplates are required to achieve this detection limit (see Sect. 10). Highly sensitive flash ATP reagents degrade their own ATP contamination. Therefore, one simply waits until the blank is gone. This is not true for extractants used to release ATP from cells, and ATP is surprisingly stable in most such solutions. There are some types of samples, such as water for injection, where the detection limit is <10 bacterial cells per 100 mL. In such conditions, one obviously has to concentrate the sample. This can be achieved in four ways:

(1) Filtration.
(2) Centrifugation.
(3) Concentration by equilibrium centrifugation in a density gradient.
(4) Concentration on the surface of magnetic nano- or micro-particles.

Filtration is feasible if the sample does not contain too many particles clogging the filter. Lack of nutrients or drying out may affect the ATP level. Centrifugation is rapid but may result in lack of nutrients or oxygen, which may lower the ATP content. Equilibrium centrifugation is a mild method. The bacteria are collected in a band, which can be sucked up and further processed. Magnetic particles are similar to centrifugation but can be more rapid and convenient. A further advantage would be to use immunomagnetic particles, making it possible to quantify individual strains rather than the total bacterial content of the sample.

2.2 Dilution

Dilution may be required if the sample matrix is highly inhibitory or if the ATP level exceeds the linear range of the assay. A certain degree of inhibition (50 % or even somewhat higher) is acceptable, provided each assay is calibrated with the

ATP standard addition technique. With this technique, the light emission is measured before and after the addition of a known amount of ATP standard (see Sect. 9). If the ATP level falls above the linear range of the assay, one may use a luciferase with a higher Km value or add a competitive inhibitor increasing the apparent Km value for ATP. However, it is usually more convenient just to dilute the sample. If the sample contains sensitive cells, such as blood cells, one should use an isotonic medium to avoid lysing the cells. One should avoid phosphate-buffered saline (PBS) as sodium chloride, phosphate, and pyrophosphate (often present as a contaminant in phosphate) are strong inhibitors of luciferase.

When assaying luciferase in vitro, one should avoid too high concentrations of cells as ATPases and pyrophosphatase from the cells may degrade ATP and pyrophosphate (the latter is present as an activator in some kits). Dilution is then an option to stabilize the light emission during the assay.

2.3 Pretreatment to Remove Specific Pools of ATP

In clinical samples such as urine, there can be three pools of ATP: extracellular ATP, ATP in mammalian cells, and ATP in bacterial cells. The extracellular ATP can be determined by just adding the ATP reagent. The mammalian ATP is determined by first incubating with an ATP-degrading enzyme and thereafter adding a strong extractant inactivating this enzyme and releasing the mammalian ATP (bacterial ATP is usually negligible compared to mammalian ATP). Bacterial ATP can be determined by first incubating with an ATP-degrading enzyme and a neutral detergent lysing the mammalian cells and thereafter adding a strong extractant releasing bacterial ATP and inactivating the ATP-degrading enzyme. After adding the ATP reagent, the light emission is measured before and after adding a known amount of ATP standard. All three assays can be performed with a single kit containing ATP-degrading enzyme, neutral detergent, strong extractant, ATP reagent, and ATP standard. The procedure for measuring bacterial ATP is described in Fig. 1.

In some cases, cells may be concentrated by filtration. In the Volvo Ocean Race 2008–2009, seven sailing boats in the race took sea water samples during the trip around the world [15]. Cells from 11.2 mL water were concentrated by filtration in a special filter holder and the extraction and assay of ATP were performed in this holder using FB12 luminometers (Berthold Detection Systems), accepting the holder in the measuring position. Chlorophyll a was measured via satellites and used to estimate the algal biomass. The slope in the log [ATP] versus log [chlorophyll a] was 0.50 and 0.64 for coastal water and open ocean, respectively. This indicates a square root relationship. The results were interpreted in terms of the spreading of organisms via ballast water.

In some situations, one may also allow the sample to degrade its own ATP. In a test for creatine kinase in dried whole-blood spots, the addition of adenosine monophosphate (AMP) allowed the adenylate kinase from the red blood cells to degrade the cellular ATP by the reaction ATP + AMP → 2 adenosine diphosphate

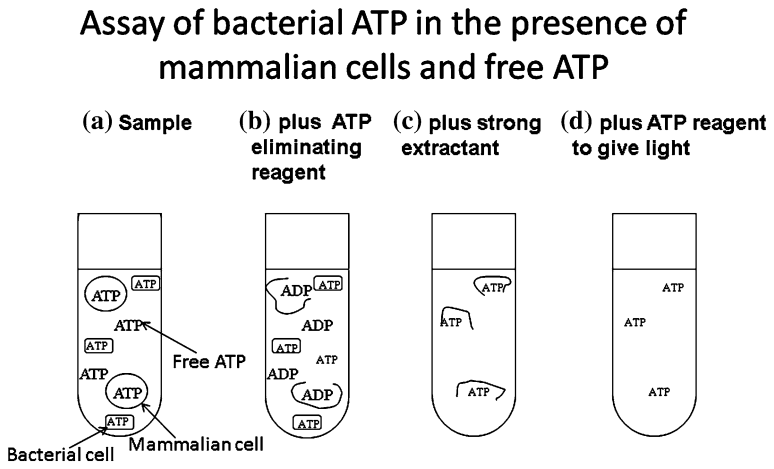


Fig. 1 Determination of bacterial ATP in **a** sample containing also free (extracellular) ATP and ATP in mammalian cells. In step **b**, the addition of an ATP-eliminating reagent lyses the mammalian cells and enzymatically degrades all nonbacterial ATP. In step **c**, enzymes are inactivated and bacterial ATP is released by a strong detergent. In step **d**, the ATP reagent is added and the light is measured before and after adding a known amount of ATP standard

(ADP). Thereafter, creatine kinase could be assayed by adding creatine phosphate and additional ADP [16].

2.4 Resuscitation

If cells have been exposed to unphysiological conditions, it may be advantageous to allow them to regain their normal ATP levels by resuscitation under suitable conditions. The total pool of ATP turns over in a few seconds, and exposing the cells to unphysiological conditions may rapidly affect their ATP levels. Resuscitation will normally allow them to build up the ATP pool again or they will die.

3 Extraction of Intracellular ATP

The extraction of intracellular ATP has two functions: (1) to open up the cell walls or membranes to release ATP; and (2) to inactivate all enzymes participating in forming or degrading ATP [1]. Because the turnover is so rapid (see Sect. 2.4), the inactivation should be immediate. The extractant must penetrate the cell walls and membranes to inactivate the enzymes. This means that ATP is simultaneously released. Furthermore, the use of strong extractants gives highly stable extracts. Normally, extracts can be stored in the refrigerator for days or in the freezer for months with no ATP degradation. It is advantageous to include

ethylenediaminetetraacetic acid (EDTA) in the extractant because ATP-degrading enzymes usually require magnesium or calcium ions.

During or after the extraction, ATP can only be degraded but not formed (ADP and AMP levels are low compared to the ATP level). Consequently, the best extractant gives the highest yield of ATP. There are two ways of showing that you have found a good extractant:

(1) With optimum levels of several extractants, the maximum ATP yield is essentially the same [17–19].
(2) The levels of ATP, ADP, and AMP are determined [17, 19] and the energy charge (EC) is calculated:

$$EC = (ATP + 0.5ADP)/(ATP + ADP + AMP) \quad (4)$$

The energy charge is normally 0.80–0.95 in healthy cells [20]. A low EC value indicates either that the extractant is poor or that the cell is physiologically stressed.

Previous studies have shown that trichloroacetic acid (TCA) is a reliable extractant [17–19]. As a reference extraction method, one simply compares the ATP yield with 10, 5, 2.5, and 1.25 % TCA in water at room temperature. The TCA concentration giving the highest ATP yield is the most reliable one. However, TCA is not so nice to work with; even when neutralized its anion is strongly inhibitory in the luciferase reaction. Consequently, extracts must be diluted, resulting in a higher detection limit. Similar ATP yields can be obtained with a quaternary ammonium compound, dodecyltrimethylammonium bromide (DTAB), and the luciferase inactivation can be obviated by complexing with cyclodextrins present in the ATP reagent [21]. At room temperature, this extractant gives close to 100 % yield with all bacterial strains tested except mycobacteria, for which the extractant should be heated to close to 100 °C [22, 23].

TCA and DTAB also work with most mammalian cells, but not with all of them [17]. Pieces of tissue must be homogenized in the presence of a strong extractant, such as TCA. DTAB can be inactivated by high concentrations of proteins or neutral detergents (Lundin, unpublished observation).

With most mammalian cells, one can use milder detergents like Triton X-100 plus EDTA provided that the cell concentration is not so high that ATPases will degrade ATP when coming into contact with the ATP reagent containing magnesium ions complexing EDTA. Extracts prepared with Triton X-100 and EDTA are not as stable as those prepared with DTAB.

4 Selection of Luciferase for Different Applications

Previously, luciferases were available from a few different species; the most popular one was luciferase from *Photinus pyralis*. Today, one can choose from a variety of recombinant luciferases with different properties. If you are skilled in

molecular biology, you can even design your own luciferase meeting your specific specifications. You must then consider which properties are the most important for a particular application.

4.1 Reporter Gene Assays

In reporter gene assays, there are obviously a number of concerns regarding the entire DNA construct introduced into the cell. Here, we will limit the discussion to enzymatic properties of the luciferase. The following parameters are of interest:

1. A high turnover number and a high quantum efficiency give more light.
2. Low Km for ATP and D-luciferin makes it easier to achieve saturating substrate concentrations, especially in vivo.
3. The color of the light is important as conventional light detectors (i.e. photomultipliers) are more sensitive to short wavelengths. On the other hand, two luciferases emitting at different wavelengths make it possible to measure two reporters simultaneously.
4. The optimum temperature of the reaction (i.e. the temperature at which the light emission is strongest) is important, particularly in in vivo imaging of whole animals. The optimum temperature may be changed both by mutation [24] and by addition of certain chemicals [25].
5. A rapid degradation of luciferase within the cell makes the reporter suitable for studying short-term effects, whereas a slow degradation makes the assay more sensitive as luciferase is accumulated in the cell. The stability may be affected both by temperature and sensitivity to proteases.
6. The choice of luciferase is also affected by whether the assay is performed in vivo or in vitro, as reaction conditions for in vitro assays may be more freely selected.

4.2 ATP Assays

In ATP assays, the choice of luciferase is affected by the following parameters:

1. The number of ATP molecules consumed per emitted photon affects the sensitivity and decay rate. Quantum efficiency should therefore be high.
2. The turnover number should be as high as possible.
3. The Km value for ATP affects the linear range. From the Michaelis–Menten equation (Eq. 2), $v/V = S/(S + \mathrm{Km})$, we see that S must be negligible compared to Km to give absolute linearity. A low Km is therefore a disadvantage when measuring high concentrations of ATP. Most firefly luciferases have a Km around 0.1 mmol/L (i.e. the assay is essentially linear up to 0.001 mmol/L). There are, however, exceptions.

4. A low Km for D-luciferin makes it less expensive to achieve saturation.
5. Resistance to luciferin analogues is an important factor. In the synthesis of D-luciferin, analogues may be formed as side products. D-luciferin is also sensitive to light and oxygen. In powder form under argon and absence of light, D-luciferin is stable for at least 2 years, even at 37 °C. In solution, D-luciferin is more stable at pH 6 than at pH 7.75, as L-luciferin is formed by racemization at an alkaline pH.
6. Light emission at low wavelengths makes light detection better (see Sect. 4.1).
7. Stability towards temperature, chemicals, and surfaces should be considered. Wild-type luciferase is rapidly inactivated above room temperature. Recombinant thermostable luciferases are commercially available. It is important to realize that thermostable luciferases usually have the same optimum temperature as wild-type luciferase. Luciferases with increased resistance to detergents have been described [26, 27]. Increased resistance to surface inactivation can be achieved with proteins such as bovine serum albumin (BSA). Temperature stability can be increased by osmolytes [28].
8. The optimum temperature of the reaction (i.e. the temperature at which the light emission is strongest) may be changed both by mutation [24] and by the addition of certain chemicals [25]. In coupled assays, it is sometimes preferable to be able to use a high temperature. In clinical chemistry, most assays are performed at 37 °C. In polymerase chain reactions and pyrosequencing, the rate as well as the probability of unspecific hybridization and secondary structure formation are reduced [29].
9. The optimum pH should be determined. Wild-type luciferase can be used between pH 6–8 with a fairly stable light emission. The light intensity is around 11 % at pH 6 and around 93 % at pH 8, as compared to the light emission at the optimum pH of 7.75 [8]. The stability of D-luciferin and the complete ATP reagent also including luciferase are better at pH 7 compared to pH 7.75. Consequently, it is better to store the reconstituted ATP reagent at pH 7 and adjust pH to 7.75 just before the assay adding a suitable buffer.
10. Resistance to ionic strength is a factor to consider.
11. Resistance to compounds in libraries used in high-throughput screening for potential drugs should be considered.

5 Luciferin

5.1 D-Luciferin

There are substantial differences in the quality of D-luciferin [30]. D-luciferin is synthesized in several steps; there are different synthetic routes, and purification may be needed after each step. Furthermore, D-luciferin is sensitive to racemization at carbon 4 in the thiazoline ring. The proton at this carbon is slightly acidic

and, when returning, it may produce L-luciferin. Consequently, racemization is considerably more rapid at alkaline pH. D-luciferin is also sensitive to light and oxidation. In air, dehydroluciferin is formed. Both L-luciferin and dehydroluciferin are strong inhibitors of the luciferase reaction. In powder form under argon, however, D-luciferin is very stable and can be kept in the dark at room temperature with no degradation for more than 2 years. Solutions of D-luciferin should be freshly made or kept in aliquots at –80 °C. D-luciferin is available as free acid (cannot be dissolved in pure water), potassium salt, and sodium salt. The best way to dissolve the free acid is to use an equimolar amount of either $KHCO_3$ or $NaHCO_3$ in water. The solution should be added in portions not to arrive at alkaline pH for long and the light should be dim. In this way, it is possible to prepare solutions containing at least 25 mg D-luciferin per milliliter. The potassium and sodium salts can be directly dissolved in water.

5.2 Luciferin Analogues and Derivatives

There are three types of luciferin analogues and derivatives that may be used for analytical purposes:

1. When performing the luciferase reaction, the analogue has some advantage from an analytical point of view in certain conditions. The analogue may be more stable or make it possible to use more favorable reaction conditions. Examples of luciferin analogues having advantages from these points of view are aminoluciferin and fluoroluciferin.
2. Inhibitory analogues or derivatives may be used to quench the light from the luciferase reaction in a dual-reporter gene assay. Competitive inhibitors can be used to increase the Km for D-luciferin, resulting in an increased upper limit for an assay of D-luciferin.
3. Enzyme substrates may be made by coupling various leaving groups to the 6′-position in the benzothiazole ring in luciferin or aminoluciferin or to the carboxyl group in luciferin. When the enzyme acts on the substrate, free luciferin/aminoluciferin is generated and can be measured by the luciferase reaction. This has been used for assays of CYP450, caspases, and other proteases [31].

6 Kinetics of the Luciferase Reaction

When optimizing assays based on the luciferase reaction, it is crucial to understand what affects the rate of the reaction (i.e. the intensity of the emitted light). The following rules should be considered:

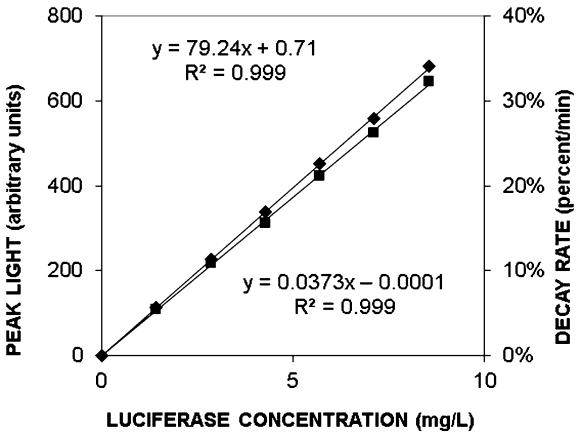


Fig. 2 *Peak* height (*diamonds*) and decay rate (*squares*) of the light is proportional to the luciferase level

1. All components in the reaction mixture except the analyte should, in principle, be present in optimal concentrations.
2. The analyte should be within the linear range determined by the Michaelis–Menten equation (Eq. 2).
3. In coupled enzymatic assays, rules 1 and 2 do not always apply because one is dealing with at least two enzymatic reactions (see Sect. 7.3).
4. Inactivation of luciferase during the measurements should be avoided because it results in complicated kinetics. Luciferase may be inactivated by the walls of the cuvette or microplate well, proteases in the sample, or detergents used for extracting bacteria. Product inhibition should not appear when the reagent is optimized for analytical purposes.

Under conditions when luciferase is not inactivated or subjected to an increasing inhibition as a consequence of accumulating product inhibition, the intensity of the light emission at low substrate concentrations is proportional to the substrate concentration. This follows from the Michaelis–Menten equation. In an ATP reagent containing luciferase and D-luciferin and a low ATP concentration ($<10^{-6}$ mol/L), the light emission is stable if luciferase consumes only negligible amounts of ATP per time unit. If, on the other hand, the luciferase activity is high, ATP will be depleted; consequently, the intensity of the emitted light will go down. In fact, the peak light emission obtained directly after adding the ATP and the decay rate of the light are both proportional to the luciferase level in the reaction mixture, as shown in Fig. 2 [32]. A high luciferase level gives a high light intensity and therefore a high sensitivity and a low detection limit. On the other hand, the decay rate is more rapid, as can be seen in Fig. 3 for three types of reagents. These three types of reagents give parallel double logarithmic standard curves (Fig. 4), which are linear from the detection limit (provided the blank is

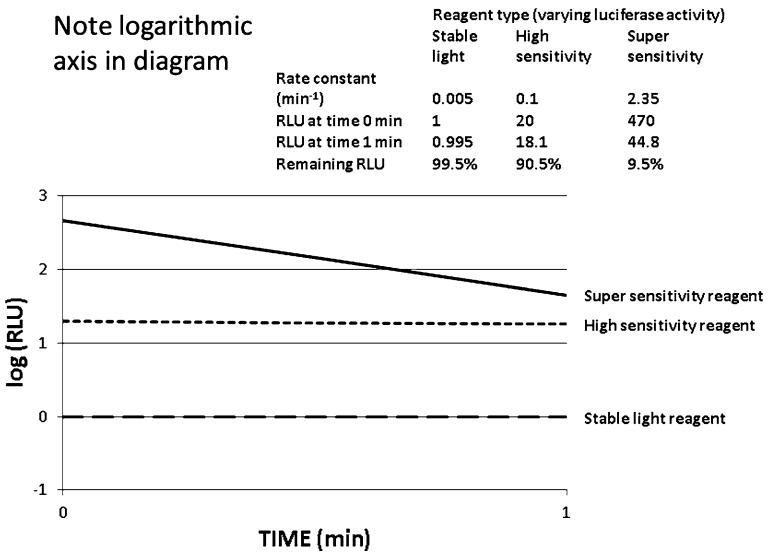
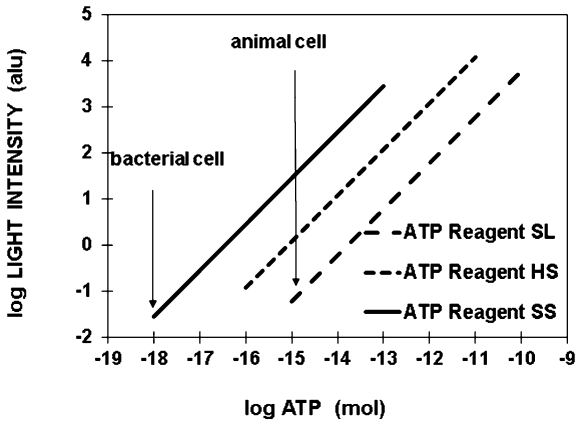


Fig. 3 Intensity and decay of light emission with various types of ATP reagents. Note the logarithmic *axis* of relative light units

Fig. 4 Double logarithmic standard *curves* for the reagents shown in Fig. 4



subtracted) to upper limit of the linear range of the luciferase reaction (0.01 × Km) or the upper limit of the linear response for the luminometer, whichever is lowest. It is obvious that one has to choose between high sensitivity and low decay rate; the two can never be achieved with the same reagent.

ATP reagents with a high luciferase activity (e.g. 90 % of the light emitted and 90 % of the ATP depleted within 1 min) are available [1]. With such reagents, the total emitted light can be extrapolated to extinction from measurements of the light emission at any two points in time. This total emitted light has the advantage that it

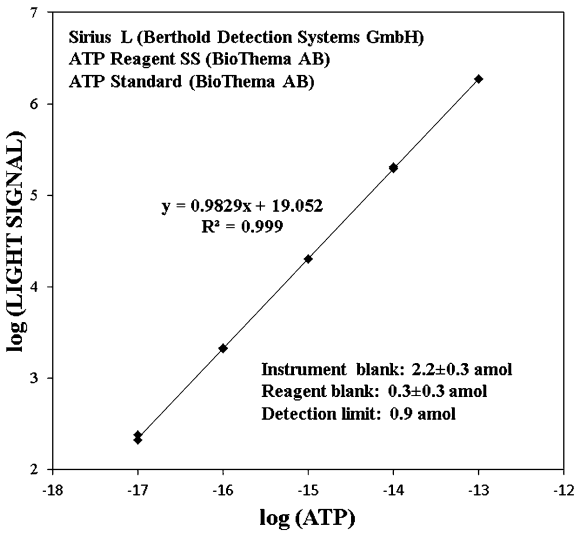


Fig. 5 Linearity and detection limit using a sensitive luminometer (Sirius L; Berthold Detection Systems GmbH) and a flash reagent (ATP Reagent SS; BioThema AB). The detection limit is calculated as three times the standard deviation of the reagent blank (0.3 amol). The constant portion of the instrument and reagent blanks are not included in this calculation because they were subtracted before plotting the data

is not affected by inhibitors changing the decay rate, provided the quantum yield is not affected [1]. A detection limit of 1 attomole has been achieved with this type of flash reagent (Fig. 5).

The major decision when optimizing an assay of luciferase is what is most important—sensitivity or stability of light emission. Just as with ATP assays, a stable light and a high sensitivity cannot be obtained with the same reagent. In assays of luciferase, the explanation is not depletion of substrates, as the decay rate in percent per minute is independent of luciferase concentration [33]. As described in Sect. 7.2, the addition of PPi strongly activates the light emission, whereas the addition of PPiase decreases the light emission to 14 % compared to the level in the presence of PPi. The addition of PPiase reduced the decay rate from 4 to 0.3–0.4 % per min [33]. This indicates that inactivation of luciferase by proteases or surface contact, which should be similar regardless of the presence of PPi, is not important. Furthermore, Gates and DeLuca could isolate the luciferase-oxyluciferin—AMP complex in the presence of PPiase [34]; however, in its absence, they could only isolate the luciferase-oxyluciferin complex without AMP. A stabilized light emission from ATP reagents can be obtained in the presence of PPi [35]. All these findings speak in favor of a role of PPi in the destabilization of the enzyme-product complex. If one lets the light emission go to 50 % of the initial level and then adds the same amount of luciferase, the light emission increases to 150 % of the initial level; that is, the first added luciferase has lost half of the activity and the second addition gives the same activity as the first one did initially (Lundin, unpublished observation). This means that the light emission is unaffected by accumulating oxyluciferin. Furthermore, this effect would be expected to be stronger at high luciferase levels, while the decay rate is the same regardless of luciferase level [33]. We have also seen that adding various inhibitors to

recombinant luciferase from *Luciola cruciata* decreases the decay rate as well as the light emission (Lundin unpublished observation). Rather, it seems as if the luciferase is worn out by the catalytic reaction as the decay rate is lower when the reaction mixture is unfavorable for the light emission.

7 Optimization of Reagents

As stated in the abstract, optimization is as important for the sensitivity as for the reliability of the assay. Small deviations of reaction conditions coming from inhibitors or variations in temperature, for example, should not affect the outcome of the assay.

7.1 Optimization of Assays of ATP

Under conditions with low concentrations of ATP and luciferin, the intensity of the light emission is proportional to the concentrations of ATP, luciferin, and luciferase. Depending on the geometry of the measuring chamber and the light detector, it is also roughly proportional to the total reaction volume, V.

$$I = k * [\text{luciferase}] * [\text{luciferin}] * [\text{ATP}] * V \tag{5}$$

A dilution of 1 % of the reaction mixture, for example, results in an effect according to the above equation of $0.99 \times 0.99 \times 0.99 = 0.97$, or a decrease of the light of around 3 %. This may have serious effects on the use of internal ATP standardization.

The luciferase concentration cannot be optimized; there is no such thing as a saturating enzyme level in a kinetic assay (cf. Sect. 4). One can, however, make sure that the luciferase activity is high enough to allow measurement of the amount of ATP that one wants to detect. This can be done by increasing the concentration of luciferase, choosing a better luciferase, and/or by optimizing conditions for the luciferase reaction. Increasing the concentration is easy but may be expensive. Choosing a better luciferase has already been discussed in Sect. 3.2. Optimizing reaction conditions can be done with respect to choice of buffer, pH, temperature, luciferin, magnesium salt, stabilizers, and activators. The buffering capacity should be adequate for the type of samples one wants to assay. It should not be higher because luciferase is sensitive to ionic strength. One should also consider the likelihood of microbial growth in the buffer. The author has never seen growth in Tris-acetate buffer, pH 7.75, supplemented with 0.5 or 2 mmol/L EDTA (not even after years at room temperature). The assay should normally be performed at the optimum pH, which may be slightly different for different buffers. The optimum temperature is generally around 23–25 °C, even for temperature-stable recombinant

luciferase. However, the optimum temperature may be changed by additives [25]. Optimizing luciferin concentration will at least remove one factor in Eq. 5 and reduce the effect of dilution to 2 %. The optimum luciferin concentration is normally around 0.7 mmol/L, but it varies for recombinant luciferases. It is not ATP but Mg-ATP that is the substrate in the luciferase reaction. The magnesium salt therefore should be in excess of the highest ATP concentration and chelators such as EDTA that may appear. On the other hand, high concentrations contribute to the ionic strength and are inhibitory. BSA is an excellent stabilizer, but levels exceeding 0.1 % in the reaction mixture are inhibitory. It may also contain enzymes that interfere with the assays.

Optimizing ATP hygiene assays for surfaces involves several problems. The swabs used today have several disadvantages:

1. They are round, whereas the surface to be sampled normally is flat. This makes it difficult to cover the entire surface (normally 10 × 10 cm).
2. They absorb only a fraction of what is on the surface because the cells are often bound to the surface.
3. The shelf life of the so-called single-shot devices is relatively short compared to reagents delivered freeze-dried in glass vials.
4. They are bulky, making transport expensive.
5. Each test cannot be calibrated by measuring the light before and after adding a known amount of ATP. Detergents or dirt left on the surface may then give a falsely low reading.
6. Different systems use different readings of relative light units for the same amount of ATP. This makes it difficult to compare results between different users.

The author has addressed the above problems using flat swabs, no pipettes, and reagents in dropper bottles, including a certified, liquid-stable ATP standard for calibration purposes. Furthermore, a test specific for bacterial ATP has been developed. When the pretreatment steps (degradation of nonbacterial ATP and release of bacterial ATP) are performed on the sampling surface, the result of the assay may be expressed in the number of bacterial cells. The test can also be used for total ATP, omitting the degradation of nonbacterial ATP. It should be mentioned that there is a test for ATP + AMP. Because AMP is formed from ATP when cells die, this test measures both living and dead cells.

7.2 Optimization of Assays of Luciferase

In the optimization of reagents for assays of luciferase, it is possible to optimize both luciferin and ATP concentrations. One may also optimize for reaction conditions and for various stabilizers and activators. Different luciferases have different optima, and one must also consider how different reporter genes and their

products behave in different cells. A major question for in vitro assays is whether stable light or maximum sensitivity is required.

In an experiment, D-luciferin, ATP, DTT, BSA, PPi, $MgAc_2$ were optimized with regard to light intensity in the assay of wild-type luciferase from *Photinus pyralis* [33]. The decay rates were measured and the ratios between decay rate and light emission were calculated. The following results were obtained with increasing concentrations of the parameter being optimized:

1. With D-luciferin, the decay rate increased but the ratio was essentially constant.
2. With PPi, the decay rate and the ratio increased at low concentrations but were essentially constant at optimum levels.
3. With ATP and $MgAc_2$, the decay rate increased but the ratio decreased when the concentration approached the optimum and was essentially constant at higher concentrations.
4. With DTT and BSA, the decay rate decreased; the ratio also decreased initially, but after passing the optimum concentration it was constant.

A decreasing ratio at low levels of ATP, $MgAc_2$, DTT, and BSA can be interpreted as a specific stabilizing effect counteracting the decay. With D-luciferin and PPi, this effect was not seen. The final assay conditions were as follows: 0.2 g/L D-luciferin, 0.2 mmol/L ATP, 20 mmol/L DTT, 2 g/L BSA, 0.02 mmol/L PPI, and 10 mmol/L $MgAc_2$. The decay rate was around 4 % per min regardless of the luciferase concentration. Interestingly, PPi had a 5.5× activating effect on the light emission. Adding PPiase to degrade all PPi resulted in only 14 % of the light emission obtained in the presence of 0.02 mmol/L PPi, while the decay rate went down to 0.3–0.4 % per min. By adding various inhibitors, it is possible to obtain a half-time of >5 h, but one must then sacrifice >90 % of the light intensity.

The luciferase molecule also influences the decay rate. The 4 % per min was obtained with *Photinus pyralis.* The decay rate with a thermostable recombinant luciferase from *Luciola cruciata* is only slightly above 1 % per min under essentially the same reaction conditions.

Using the 5′-fluoroluciferin analogue, the pH optimum is changed from 7.8 to 7.0 [36]. The decay rate and the light emission is somewhat lower than for reagents optimized with respect to sensitivity, but the sensitivity is better than for reagents optimized for stable light emission. The reagent is more stable than those based on D-luciferin as the pH is lower.

7.3 Optimization of Coupled Enzyme Assays

In coupled assays based on the firefly luciferase reaction, either luciferin or ATP is formed in one or several coupled enzymatic reactions. The formation of the substrate (luciferin or ATP) or depletion of ATP may either be performed in a separate step followed by reading the amount formed by subsequently adding the reagent containing luciferase or by having the luciferase present during the

formation or depletion of the substrate, continuously monitoring the formation or depletion by measuring the light intensity. In the latter case, the luciferase level should be low and should consume only a negligible proportion of ATP or luciferin.

Conditions for continuous monitoring of ATP in assays of enzymes and metabolites were first described in the 1970s [8]. Reagents have since then been improved by using recombinant thermostable luciferase and high-quality luciferin. The rate of ATP formation may be used in kinetic assays of enzymes, such as CK [10–14], oxidative phosphorylation [37–40] and photophosphorylation [41–45]. The rate of ATP depletion may be used in assays of protein kinases [46], aminoacyl-tRNA synthetases, and ATPases. The rate of ATP depletion may be used in kinetic assays of metabolites, such as a glycerol assay using the glycerol kinase reaction [47, 48] and an assay of urea using ATP-hydrolyzing urease [49]. Endpoint assays of metabolites, in which one allows the formation of ATP to run to completion, are also possible, such as assays of ATP/ADP/AMP in a single cuvette [17, 19] and inorganic pyrophosphate [50]. The latter assay was later used in the development of pyrosequencing [51, 52].

7.3.1 Endpoint Assays of Metabolites

In endpoint assays of metabolites, optimization is normally not that critical. One can simply add more enzyme to rapidly reach the endpoint. In the assay of ATP/ADP/AMP, we used the following reactions:

$$\mathrm{ADP} + \mathrm{phosphoenolpyruvate} \overset{\mathrm{pyruvate}}{\longrightarrow} \mathrm{kinase ATP} + \mathrm{pyruvate} \tag{6}$$

$$\mathrm{AMP} + \mathrm{CTP} \overset{\mathrm{adenylate}}{\longrightarrow} \mathrm{kinase ADP} + \mathrm{CTP} \tag{7}$$

The pyruvate kinase (PK) reaction is straightforward. With the low ATP and AMP concentrations from the sample (far below the Km values), the adenylate kinase (AK) reaction would have been very slow in the absence of cytidine triphosphate (CTP). When CTP was added in a final concentration of 2.5 mmol/L, the reaction reached the endpoint within 5 min, irrespective of ATP and AMP concentrations. The assay takes advantage of the nonspecificity of the ATP binding site in AK and the specificity of the ATP site in luciferase.

The calibration of the certified ATP standard is based on an endpoint assay using the hexokinase reaction and a stable light-emitting ATP reagent:

$$\mathrm{ATP} + \mathrm{glucose} \overset{\mathrm{hexokinase}}{\longrightarrow} \mathrm{ADP} + \mathrm{glucose} - 6 - \mathrm{phosphate} \tag{8}$$

A mixture was prepared to contain approximately 25 μmol/L ATP (from the unknown stock solution), 1.25 mmol/L AMP, 1.25 mmol/L PPi, 0.1 M Tris, and 2 mmol/L EDTA at a final pH 7.75. Half of the mixture was supplied with 20 μmol/L glucose standard (purity 99.9 %), such that approximately 80 % of the ATP would be depleted. Four cuvettes containing 0.8 mL of each of the two mixtures were placed in the instrument and 0.2 mL stable light ATP reagent with 50 U/mL hexokinase was added. Light signals between 6 min (the endpoint) and 15 min were extrapolated to time zero (i.e. $I_{-\text{glucose}}$ and $I_{+\text{glucose}}$). The unknown ATP concentration was calculated from the known glucose concentration (20 μmol/L) by multiplying it with $I_{-\text{glucose}}/(I_{-\text{glucose}} - I_{+\text{glucose}})$. After correction for dilutions, the result from four determinations was 2.052 ± 0.003 mmol/L (μ ± σ).We used rather high concentrations of ATP and glucose to reach the endpoint as quickly as possible. The ATP concentration would actually have been outside the linear range of the luciferase reaction, if the competitive inhibitor AMP had not been included. PPi was included to stabilize and decrease the light emission to make sure that the light was essentially constant and within the linear range of the 1251 Luminometer (BioOrbit Oy, Turku, Finland), which was adjusted to 25 ± 0.1 °C. A constant temperature is essential to achieve a high accuracy.

7.3.2 Kinetic Assays of Metabolites

The kinetic assay of glycerol is based on the following reaction:

$$\text{ATP} + \text{glycerol} \overset{\text{glycerol}}{\longrightarrow} \text{kinaseADP} + \text{glycero} - 1 - \text{phosphate} \qquad (9)$$

The rate of ATP depletion in the glycerol kinase (GK) reaction is measured by continuously measuring the decay of the light from the luciferase reaction [47, 48]. The pH optimum of the GK reaction is 9.8, a pH where we could not run the luciferase reaction, so it was decided to use pH 7.75, the optimum for luciferase. The lower GK activity was compensated by adding as much GK as was required to get an easily measured decay rate for the light in the dynamic range for glycerol.

The ATP concentration could be 0.01, 0.1, or 1 μmol/L (all concentrations well below the Km values for GK and luciferase) and the decay rate of the light was the same and depended only on glycerol concentration and GK activity. The Km of GK was 40 μmol/L and would give a linear range only up to 2 μmol/L. This problem was solved by determining V and Km and plotting the standard curve as v/V versus $S/(S + \text{Km})$, where v is the decay rate in percent per minute and S is the glycerol concentration. Thus, the left-hand side of the equation was plotted against the right-hand side, which means a straight line from the point (0, 0) to (1, 1). In this way, the dynamic range was extended to give a linear plot in the interval 0.07–100 μmol/L [47, 48]. The plot could also be made in a log-log version, which was convenient as it covered three orders of magnitude. The reproducibility was

very good, giving a between run CV of <2 %. The method was, and still is, used to measure the release of glycerol from human fat cells in studies of hormonal effects on lipolysis rate. As fat cells float on top, it was easy to collect a sample from the below liquid containing the released glycerol.

Urea can be assayed using urease [urea amidohydrolase (ATP-hydrolysing); EC 3.5.1.45] and firefly luciferase [49]. The reaction is as follows:

$$\mathrm{ATP} + \mathrm{urea} \xrightarrow{\text{urease}} 2\mathrm{NH_3} + \mathrm{CO_2} + \mathrm{ADP} + \mathrm{P}_i \tag{10}$$

The assay looks similar to that of glycerol [47, 48], but it is actually much more difficult. The major reason is that this urease has a side reaction acting as an ATPase (v_{blank}). This reaction could be partially counteracted by various alcohols, which also affected Km and *V*. It was finally decided to use 1, 2-propandiol. The number of parameters made it necessary to use multivariate analysis to optimize the assay. This was done in three experiments:

1. K^+, $NaHCO_3$, pH, $MgAc_2$, 1, 2-propandiol
2. Chloride/acetate, Na^+/K^+, Mg^{2+}, 1, 2-propandiol
3. K^+, Mg^{2+}, 1, 2-propandiol

In total, 32 reaction mixtures were assayed. The most important parameters turned out to be K^+, Mg^{2+}, and propandiol and response surface plots showed that the widest dynamic range of the assay was obtained with 50 mmol/K^+, 16 mmol/L Mg^{2+}, and 2.5 mol/L 1, 2-propandiol. The optimization led to an increase of the V/v_{blank} ratio by a factor of 29. It was a first order reaction, which could be plotted in the same way as the glycerol assay—that is, v/V versus $S/(S + \mathrm{Km})$. The assay range was 0.1–50 nmol urea. It had a good correlation (R = 0.978) with a spectrophotometric method. The experimental design using the multivariate analysis allowed us to optimize this assay in just a few weeks. The traditional chessboard design would have taken a much longer time.

7.3.3 Kinetic Assays of ATP-Forming Enzymes

In kinetic assays, reaction conditions can be optimized with respect to:

1. The luciferase reaction
2. The coupled enzyme reaction
3. The combined reaction resulting in the highest light emission

The first alternative is not logical because luciferase is just an auxiliary enzyme. The second alternative has the advantage that the coupled enzyme is the analyte. The third alternative gives the highest sensitivity. When we developed the first kit for clinical chemistry based on bioluminescence [14], CK, we had to choose. Already available were recommendations from several societies for clinical chemistry based on optimization work performed in several countries on the

spectrophotometric assay of CK [53]. This optimization was based on alternative 2, as the two auxiliary enzyme reactions were not rate limiting. In the bioluminescent assay, however, CK as well as luciferase are rate limiting for the light emission. We decided to follow the recommendations of the clinical chemistry societies as far as possible. Consequently, we used the same buffer, the same pH, the same magnesium concentration, the same *N*-acetylcystein concentration, and the same EDTA concentration. Lower concentrations were used for ADP and diadenosine phosphate (DAPP; an inhibitor of adenylate kinase). The reason was that there is something in these nucleotides that gives light emission in the luciferase reaction, which could not be completely removed by ion exchange chromatography or enzymatic degradation of ATP. We used a lower concentration of AMP (an adenylate kinase inhibitor) as AMP is a strong inhibitor of luciferase. A lower concentration of creatine phosphate was used for the same reason.

Because the luciferase activity may change from sample to sample, all assays ended with measuring the light before and after adding a known amount of ATP standard. The spectrophotometric CK assay can be performed at 25, 30, or 37 °C. For the bioluminescent assay, we chose 25 °C because the wild-type luciferase enzyme was inactivated at higher temperatures. The spectrophotometric and bioluminescent CK assays were later evaluated and found to have a very good correlation and (in spite of slightly different substrate concentrations) almost identical results [54]. The major difference was that the bioluminescent assay had a much better detection limit. This allowed measurement of CK-B (i.e. the heart-specific CK activity), even in healthy patients [10]. In a group of patients admitted to the coronary care unit, a discrimination level of an increase of CK-B > 5 U/L patients with acute myocardial infarction could be completely separated from patients without this condition, according to conventional criteria [10].

The assay of CK was later optimized for screening for Duchenne muscular dystrophy [16]. This disease affects 1 in approximately 3,600 newborn boys (girls are not affected but can be carriers) and results in degradation of the muscles and death around 25 years of age. The degradation results in increased CK levels in the blood. The assay was used in a voluntary screening program to avoid a second birth of a boy with Duchenne muscular dystrophy in a family already having an affected boy. Samples were collected a few weeks after birth as a drop of blood on a filter chapter and sent to CK-Test Laboratorium (Breitnau, Germany). The dried blood contained a lot of ATP from the blood cells. This ATP was depleted using the adenylate kinase from the blood itself and added AMP. The assay has been used in more than half a million tests with very good results. A cure by gene therapy is now in clinical trials. When available, this will result in a need for screening all newborn boys and also for therapeutic monitoring. A higher concentration of DAPP was needed in this assay because the adenylate kinase was higher than in serum.

7.3.4 Kinetic Assays of ATP-Depleting Enzymes

A real-time ATP depletion assay was developed for measuring protein kinase activity in high-throughput screening (HTS) of compound libraries [46]. There are some 500 different protein kinases involved in the regulation of various cell functions. Consequently, the pharmaceutical industry is interested in finding drugs that inhibit specific kinases. Different protein kinases have different pH optima, so we decided to use the optimum for luciferase. Actually, we thought that we could take the conditions from the glycerol assay described above. This assay is performed in 1 mL and that worked well also for protein kinase. Our protein kinase collaborators told us, however, that protein kinases as well as their substrates are extremely expensive and screening a library with perhaps a million compounds would be out of the question in 1 mL reaction volume. Therefore, we tried to go down in volume, but this caused a rapid decay of the light, even in the absence of ATP depletion. Luciferase was inactivated by the surfaces of the cuvette or well. The ratio between the surface and the liquid volume increases dramatically when going from 1 mL to 50 μL, for example. Consequently, a larger proportion of the luciferase molecules hits the surface and is inactivated per time unit. Obviously, we needed additives to the buffer to stabilize against surface inactivation. Finally, we developed a buffer resulting in half-time of the light emission for the blank (no ATP depletion) of 8–9 h in a 50-μL total reaction volume. (Most likely, one can go down in volume even further if one has the appropriate equipment). In order to obviate even the effect of this remaining inactivation, the light readings were normalized with the corresponding blank value at each point in time. In this way, we got completely straight lines for log values of the normalized light versus time as expected in a first-order reaction. The slope of these lines (the rate of the ATP depletion) was proportional to the kinase concentration and formed a perfectly straight line when plotted as a log-log graph. In HTS, the quality of an assay is expressed as the Z′-factor, which for an excellent assay should be 0.5–1.0. The protein kinase assay had a value of 0.96, which was reached after a fivefold reduction of the ATP concentration. The assay does not require continuous monitoring of the light; it is enough to measure the light at any two points in time. This also means that the initial ATP concentration (1 μmol/L) is not critical. Furthermore, it does not matter if the luciferase activity is partially inhibited, as this affects the light emission equally at both points in time. Compound libraries contain around 3 % luciferase-inhibiting compounds [55], which may not be classified if the measured light falls below the control (i.e. neither inhibition of kinase nor of luciferase). In this case, a renewed assay with another method must be performed. This is the way other ATP depletion assays are performed. The kit developed as described with two light readings is therefore much more robust than when only reading once. The assay is linear for kinase over at least three orders of magnitude. The kit is not restricted to assay of protein kinases, but for all ATP depletion assays, such as ATPases and aminoacyl-tRNA synthetases.

8 Stabilization of Reagents

Wild-type luciferase is fairly unstable and is rapidly inactivated above 25 °C. Luciferase stability may be improved by various additives. BSA, some neutral detergents, and osmolytes are examples. Furthermore, there are recombinant thermostable mutants of recombinant luciferase [56, 57]. Such mutants may even be used to prepare liquid-stable ATP reagents with only a moderate loss of activity when stored in the refrigerator for 1 year. This is obviously an important advantage to the manufacturer, who can skip the expensive and time-consuming lyophilization step. Furthermore, it is a great advantage to the user, as the reconstitution step and subsequent loss of activity over time is avoided.

D-luciferin is sensitive to light, oxygen, and alkaline pH. When exposed to these agents, inhibitors of the luciferase reaction, such as dehydroluciferin and L-luciferin, are formed. These inhibitors decrease the light emission even when present at very low concentrations (<1 % compared to D-luciferin). A statement that the D-luciferin was >99 % pure by high-performance liquid chromatography (HPLC) is therefore not a guarantee of high quality. In a comparison of D-luciferin from eight manufacturers using HPLC and biochemical performance (the measurement of the light emission), there was no correlation between the two measures [30]. Even the preparation that had only 77 % activity did not seem more contaminated than preparations with higher activity.

From a stability point of view, ATP reagents should be stored at pH 6.5–7.0. If the reagent is only weakly buffered, the pH may be brought to the optimum pH 7.75 by a strong buffer in the extractant or in some other auxiliary reagent, for example.

9 Calibration of Assays

ATP assays are often reported in relative light units (rlu). The light signal reported by the luminometer is, in addition to the ATP concentration, influenced by the following parameters:

1. The sample matrix affects the luciferase activity and therefore the light intensity. If the sample matrix is not the same in all samples, comparisons between samples cannot be made even within an experiment.
2. Luciferase activity varies between different production lots.
3. Luciferase activity changes with time as the reagent ages.
4. Luciferase activity is temperature dependent. Having samples and reagents at different temperatures will cause erroneous results. A temperature deviation of ±5 °C from the optimum temperature gives around 15 % less light.
5. Turbidity in the samples decreases the light reaching the detector. Although turbidity is less of a problem with luminescence measurements as compared to spectrophotometry, one still has to compensate for it.

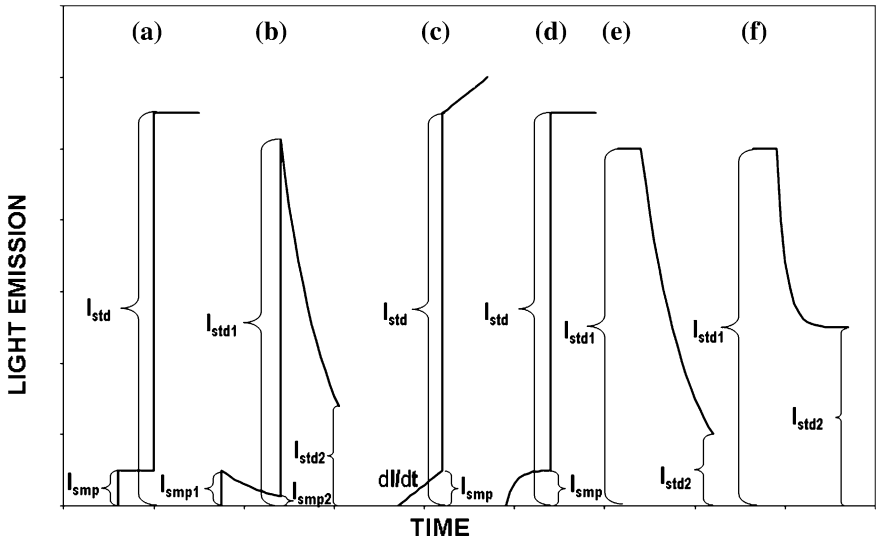


Fig. 6 The calibration of assays by the standard addition technique in various types of assays. **a** ATP assay with stable light reagent **b** ATP assay with flash reagent **c** Kinetic assay of enzyme or metabolite (ATP formation) **d** Endpoint assay of metabolite (ATP formation) **e** Kinetic assay of enzyme or metabolite (ATP depletion) **f** Endpoint assay of metabolite (ATP depletion)

6. The material and design of the cuvette or microwell affects the light that reaches the detector.
7. The geometry of the light-measuring chamber is often such that only a small percentage of the light actually reaches the detector. Positioning of the light-emitting solution is therefore important. For the same reason, the volume of the reaction mixture must be the same in all samples.
8. Only a percentage of the photons reaching the detector are registered. Furthermore, the percentage depends on the wavelength of the light. If the reaction conditions cause a red shift, the signal will be lower, even if the same number of photons reaches the detector.
9. Photomultiplier sensitivity is affected by high voltage supply, temperature, and magnetic fields.
10. The electronics after the detector may change the way the data are presented.

ATP assays are often calibrated by referring to a standard curve. Provided such a curve is prepared every time an assay is performed, it takes away some of the problems. However, neither the effect of the sample matrix nor that of varying turbidity among samples are compensated for by a standard curve.

A more reliable method of calibrating ATP assays is the standard addition technique. The various ways of using the standard addition technique are illustrated in Fig. 6. The techniques and formulae apply only when there is no gradual inactivation of luciferase during the duration of the measurement. Except for the

situation in Fig. 6b, the ATP depletion in the luciferase reaction is negligible. For ATP measurements with stable light-emitting reagents (e.g., Fig. 6a), the procedure is as follows:

1. Add a known volume of sample to the ATP reagent and measure the light signal, I_{smp} (rlu).
2. Add a known volume of an ATP standard to the same cuvette and measure the light, I_{std} (rlu).
3. Calculate the unknown ATP amount in the sample, ATP_{smp}, from the known amount in the ATP standard added, ATP_{std}, using the following equation:

$$ATP_{smp} = I_{smp}/(I_{std} - I_{smp}) \times ATP_{std} \tag{11}$$

This formula applies only when the light emission from the sample is essentially constant (i.e. when ATP depletion in the luciferase reaction is negligible). When this is not so, the procedure is as follows (cf. Fig. 6b):

1. Add a known volume of sample to the ATP reagent and measure the light signal, I_{smp1}, as soon as possible.
2. Measure the light again, I_{smp2}, just prior to adding the ATP standard.
3. Add a known volume of an ATP standard to the same cuvette and measure the light, I_{std1}.
4. Calculate the unknown ATP amount in the sample, ATP_{smp}, from the known amount in the ATP standard added, ATP_{std}, using the equation:

$$ATP_{smp} = I_{smp1}/(I_{std1} - I_{smp2}) \times ATP_{std} \tag{12}$$

The above procedure can be used when reading all wells in a microplate before adding the ATP standard. Thereafter, one should go back reading well by well before (I_{smp2}) and after (I_{std1}) adding the ATP standard; that is, both readings are made in each well before going to the next well.

Not all microplate luminometers can be programmed to perform steps 2 and 3 in one reading cycle. In this case, one can read I_{smp1} for all wells, then add the ATP standard measuring I_{std1}in one step for each well, and finally make a last reading for all wells measuring I_{std2} (cf. Fig. 6b). The decay rate of the light (percent per minute) is the same before and after adding the ATP standard. Thus, if the times between all three readings (I_{smp1}, I_{std1}, and I_{std2}) are the same, then $I_{smp2} = (I_{std2}/I_{std1}) \times I_{smp1}$. The formula is therefore:

$$ATP_{smp} = I_{smp1}/(I_{std1} - \left(\frac{I_{std2}}{I_{std1}}\right) \times I_{smp1}) \times ATP_{std} \tag{13}$$

Figure 6c shows the time course of an ATP-forming reaction in a kinetic assay of an enzyme or metabolite performed with a stable light ATP reagent. The rate of the increase of the light, *dI/dt* (rlu/minute), is measured and divided by the increase

of the light obtained when adding the ATP standard, I_{std} (rlu). The rate of ATP formation in moles per minute, $d\text{ATP}/dt$ (moles/minute), is calculated as:

$$\frac{d\text{ATP}}{dt} = \frac{dI}{dt} / (I_{std} - I_{\text{smp}}) \times \text{ATP}_{\text{std}} \tag{14}$$

Figure 6d shows the time course of an endpoint assay of a metabolite based on an ATP-forming reaction and a stable light ATP reagent. The amount of ATP formed is calculated by the equation:

$$\text{ATP}_{\text{smp}} = I_{\text{smp}} / (I_{\text{std}} - I_{\text{smp}}) \times \text{ATP}_{\text{std}} \tag{15}$$

A kinetic assay of an enzyme or metabolite based on an ATP-depleting reaction (Fig. 6e) is best set up as a first-order reaction:

$$[\text{ATP}_t] = [\text{ATP}_0] \times e^{-kt} \tag{16}$$

With a stable light ATP reagent, the light emission is proportional to ATP concentration and the rate of the reaction, k (min^{-1}), can be measured as:

$$k = [\ln(I_0) - \ln(I_t)]/t = [\ln(I_0/I_t)]/t \tag{17}$$

Actually, the light emissions, I_0 and I_t, can be chosen anywhere on the decay curve as the rate k is the same everywhere.

An endpoint assay of a metabolite based on an ATP-depleting reaction and a stable light ATP reagent (Fig. 6f) starts by adding the ATP standard, measuring the light, I_{std1}, and then adding the enzyme measuring the decreased light emission, I_{std2}. The metabolite concentration is calculated from the amount of depleted ATP as follows:

$$\text{ATP}_{\text{depleted}} = \frac{I_{\text{std1}} - I_{\text{std2}}}{I_{\text{std1}}} \times \text{ATP}_{\text{std}} \tag{18}$$

There are a number of prerequisites that must be fulfilled to allow the standard addition calibration:

1. The ATP standard concentration and stability must be known. There is a certified liquid-stable ATP standard available on the market [58].
2. Luciferase activity should not be affected by an increasing inhibition or inactivation during the measurement.
3. The reaction conditions should not be affected by the addition of the ATP standard (see Eq. 5). Consequently, the volume added must be low compared to the total reaction volume (ideally <1 %). This may be difficult to achieve with retained precision, especially when using microplates. The ATP standard should be in a similar buffer as the reaction mixture in order not to change pH or ionic strength.

4. In the assays depicted in Fig. 6a–d, the amount of ATP standard added should be high compared to the sample ATP as the denominators in Eqs. 11–15 otherwise are strongly affected by the dilution effect. The dilution effect on ATP, luciferase, and luciferin concentrations can be calculated from Eq. 5. It can also be experimentally determined by adding the same volume of buffer as used for the ATP standard. The effect of diluting inhibitors in the reaction mixture can, however, not be estimated in this way.
5. After adding the ATP standard, the total initial ATP concentration should be within the linear range of the assay. The light emission is proportional to the ATP concentration up to 1 μmol/L (0.01 × Km) for most luciferases.
6. The light emission should be within the linear range of the luminometer. Most luminometers have a linear range covering several orders of magnitude. The upper limit of the linear range of the luminometer should be determined, as this is not always stated by the manufacturer.
7. In kinetic assays of enzymes or metabolites based on ATP depletion (Fig. 6e), the ATP concentration should be well below the Km values of both luciferase and the enzyme for which the rate is measured. The rate, measured as the rate constant k, is under these conditions independent of ATP concentration (cf. Sect. 7.3.2 and 7.3.4).
8. In endpoint assays of metabolites based on ATP depletion (Fig. 6f), the ATP concentration should be well below the Km of luciferase but preferably only somewhat higher than the metabolite concentration. A good accuracy is achieved if 10–95 % of the ATP is degraded.

When the dilution effect is too strong, the standard addition calibration cannot be used. It is then possible to perform the assay in two wells—one well without and one well with ATP standard—thus compensating for the volume of ATP standard by using a lower volume of buffer in the well with ATP standard.

Assays of luciferase can be calibrated using a luciferase standard, which must be of the same type as used in the assays.

10 Sources of Background and Ways to Reduce Them

The vital role of ATP in all living cells means that ATP can be found almost anywhere, at least in low concentrations. ATP is also a fairly stable molecule under the right conditions. Liquid ATP standards can be kept in the refrigerator for 2 years with essentially no degradation [58]. On the other hand, after coming into contact with ATP-degrading enzymes, ATP may disappear within seconds. Examples of ATP-free items include the following:

1. Molded plastic and glass, provided the items have been protected from contact with human hands (ATP is destroyed during the molding process).
2. Ultrapure water and organic solvents, such as ethanol.
3. Biochemicals and chemicals that degrade ATP.

Examples of ATP contaminated items that may be found in the laboratory:

1. Pipette tips, especially if they during packing or use have been in contact with human hands or other ATP-contaminated items.
2. Cuvettes and microplates not coming directly from the molding process and being contaminated afterwards.
3. Glassware not coming directly from the molding process and being contaminated afterwards.

There are several ways to avoid or reduce background that may influence the assays:

1. Always wear disposable gloves without powder (the powder contains ATP). Change frequently and do not touch anything that may be ATP contaminated. With some very sensitive luminometers and plastic cuvettes, there may be some form of electronic excitation that emits light if the cuvette has been touched by a hand with a glove. In this case, wear a glove only on one hand.
2. Cuvettes and microplates may also show a phenomenon called delayed fluorescence. After exposure to light, they emit light for some time. In this case, wait until the emission ceases before injecting the ATP reagent. Keep the lights in the laboratory low if a low detection limit is required.
3. Plastic items can be treated with an ATP-degrading enzyme to remove most of the ATP contamination. The enzyme must thereafter be inactivated.
4. Heating glass cuvettes at 275 °C for 4 h reduces the blank to 0.3 ± 0.3 attomole (Fig. 5) corresponding to a detection limit of 0.9 attomole.
5. Commercially available ADP usually contains 1–2 % ATP. This ATP may be removed by ion-exchange chromatography [11]. It may also be degraded by hexokinase plus glucose [39]. It seems, however, that we can only reach an ATP/ADP ratio of 0.002 %. Similar results have been achieved with hexokinase as well as other ATP-degrading enzymes (Lundin, unpublished observation). Whether this remaining light comes from ATP not being degraded for some reason or from some analogue not reacting with hexokinase but being a substrate for luciferase is not known. A similar problem is seen with DAPP [13]. Both ADP and DAPP are needed in the assay of creatine kinase. The same problem is not seen with AMP.
6. Flash-type ATP reagents degrade their own ATP contamination following first-order reaction kinetics, $ATP_t = ATP_0 \times e^{-kt}$, where k is the rate constant and t is the time. If $k = 0.1$ (ATP depletion is 10 % per min), $t_{1/2}$ is 23 min. If $k = 2.35$, only 10 % of the ATP is left after 1 min [1]. This makes it easy to work with flash-type ATP reagents. If the user happens to contaminate the reagent, all that is required is just to wait until the background has disappeared.

In a straightforward assay of luciferase, one rarely has problems with background. The reason is of course that firefly luciferase is only found in certain insects, which are not usually found in the laboratory. In dual assays, we can inhibit or inactivate the firefly enzyme before we measure the other enzyme, such as Renilla. When trying to simultaneously measure several luciferases with

different emission spectra, there is a significant overlapping of the spectra, even when using optical filters. This problem may be overcome to a large extent by determining calibration constants and using mathematical formulae for separating the green and red signals.

11 Effect of Measuring Different Light Parameters/Read-Outs

In general, bioluminescence is measured as the intensity of the emitted light—that is, the number of photons emitted per minute or second. A standard curve can then be plotted of light intensity versus analyte concentration. Even when the light intensity is integrated for some seconds, what is measured is essentially the light intensity. There are, however, alternatives.

With flash-type reagents, one can measure peak light, I_0, and decay rate, k. From these two parameters, one may calculate the total emitted light integrated from time 0 to time ∞, $I_{tot} = I_0/k$. Provided the quantum yield is constant, I_{tot} is unaffected by inhibition or by temperature effects as I_0 and k are affected in the same way [1]. The k value can be measured between any two times on the light curve because it follows first order kinetics. Thus, $k = (\ln I_{t1} - \ln I_{t2})/(t_2 - t_1)$.

An assay of protein kinase has been developed, which is influenced neither by ATP concentration nor by luciferase inhibition [46]. The assay has an extremely high Z′ value (0.96) and is highly suitable for HTS. It works with an ATP reagent with stable light emission and a low ATP concentration far below the Km of luciferase and kinases (1 μmol/L). Under these conditions, the kinase activity is proportional to the rate constant k, which as above can be calculated as $k = (\ln I_{t1} - \ln I_{t2})/(t_2 - t_1)$. If luciferase is inhibited by compounds in the library, it affects both light measurements, I_{t1} and I_{t2}, equally. Consequently, k is not affected at all as $(\ln I_{t1} - \ln I_{t2}) = \ln (I_{t1}/I_{t2})$. This is a considerable advantage because compound libraries contain around 3 % luciferase inhibitors [55]. Furthermore, HTS projects are time-consuming, and neither aging of the ATP reagent nor different luciferase activity in the lots will affect the measurement. The only disadvantage is that the plate has to be read twice. If one reads it only once, the results are affected by luciferase inhibition; if the reading is below that of the control (no compound added), the result cannot be interpreted. Thus, a second measurement preferable with another assay has to be performed.

12 Conclusions

When setting up assays based on the firefly luciferase reaction, one should never forget that the intensity of the emitted light is a measure of the rate of the reaction. In other words, you are dealing with a kinetic assay even when you do endpoint

assays of metabolites. Kinetic assays should always be individually calibrated because there may be sample matrix effects on the rate of the reaction. The use of the ATP standard technique solves this problem when used in a correct way. When developing a new assay based on firefly luciferase, one should consider how to select luciferase, luciferin, stabilizers, buffers, auxiliary enzymes, and metabolites. The choice is sometimes between sensitivity and robustness. Furthermore, there may be practical issues, such as the purity and price of the components. Speed and convenience are important factors. Although stable light reagents are convenient for coupled assays of enzymes and metabolites, maximum sensitivity for ATP can only be achieved with flash reagents. The latter reagents may be difficult to work with as there are ATP contaminations everywhere. A compromise between stable light and flash reagents is often preferable in the detection of bacteria. New recombinant luciferases with increased thermostability and temperature optimum or variation in emission spectrum broaden the applicability of luciferase-based assays. Luciferin analogues and derivatives have the same effect. Miniaturization has opened up the possibility for high-throughput screening. Food and hospital hygiene are important tools for avoiding the spread of infectious diseases. What is lacking is a general and practical tool for identifying different bacterial strains based on immunology, which indicates that there is still much to be investigated in this constantly developing field.

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Part II
Applications of Bioluminescence in Environment and Security

Evaluation of the Ecotoxicity of Pollutants with Bioluminescent Microorganisms

Francisca Fernández-Piñas, Ismael Rodea-Palomares, Francisco Leganés, Miguel González-Pleiter and M. Angeles Muñoz-Martín

Abstract This chapter deals with the use of bioluminescent microorganisms in environmental monitoring, particularly in the assessment of the ecotoxicity of pollutants. Toxicity bioassays based on bioluminescent microorganisms are an interesting complement to classical toxicity assays, providing easiness of use, rapid response, mass production, and cost effectiveness. A description of the characteristics and main environmental applications in ecotoxicity testing of naturally bioluminescent microorganisms, covering bacteria and eukaryotes such as fungi and dinoglagellates, is reported in this chapter. The main features and applications of a wide variety of recombinant bioluminescent microorganisms, both prokaryotic and eukaryotic, are also summarized and critically considered. Quantitative structure-activity relationship models and hormesis are two important concepts in ecotoxicology; bioluminescent microorganisms have played a pivotal role in their development. As pollutants usually occur in complex mixtures in the environment, the use of both natural and recombinant bioluminescent microorganisms to assess mixture toxicity has been discussed. The main information has been summarized in tables, allowing quick consultation of the variety of luminescent organisms, bioluminescence gene systems, commercially available bioluminescent tests, environmental applications, and relevant references.

Keywords Bioluminescent microorganisms · Environmental monitoring · Genetically modified microorganisms · Mixture toxicity · Pollutants · Toxicity bioassays

F. Fernández-Piñas (✉) · I. Rodea-Palomares · F. Leganés · M. González-Pleiter · M. Angeles Muñoz-Martín
Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain
e-mail: francisca.pina@uam.es

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Abbreviations

ASTM	American Society for Testing and Materials
BETEX	Benzene, toluene, ethylbenzene and xylene
CA	Concentration addition
CCD	Charge-coupled device
CHAs	Chlorinated aliphatic hydrocarbons
CI	Combination index
DCP	Dichlorophenol
EC_{50}	Effective concentration of pollutant that inhibits the toxicity endpoint by 50 %
GC-MS	Gas chromatography-mass spectrometry
HA	Humic acids
HOMO	Highest occupied molecular orbital
HPLC-MS	High-performance liquid chromatography-mass spectrometry
IA	Independent action
ICP-MS	Inductively coupled plasma-mass spectrometry
INFCIM	Integrated fuzzy concentration addition-independent action model
ISO	International Organization for Standardization
K_{ow}	*n*-octanol water partition coefficient
LC-MS	Liquid chromatography–mass spectrometry
LUMO	Lowest occupied molecular orbital
MOA	Mechanism of toxic action
MODEL KEY	Models for assessing and forecasting the impact of environmental key pollutants of freshwaters marine ecosystems and bioavailability
NOECs	No observed effect concentrations
PAHs	Policyclic aromatic hydrocarbons
PCP	Pentachlorophenol
PVA	Polivinyl alcohol
TBT	Tributyltin
TCA	Trichloroethane
TCE	Trichloroethene
TCP	Trichlorophenol
TEQs	Toxic equivalency factors
TPT	Triphenyltin
TU	Toxic units
OCDE	Organization for economic cooperation and development
QSAR	Quantitative structure-activity relationship
USEPA	U.S. Environmental Protection Agency
WARUS	Weak acid respiratory uncouplers

Contents

1 Introduction 67
2 Naturally Bioluminescent Microorganisms: Their Use in Environmental Monitoring 68
3 Recombinant Bioluminescent Microorganisms: Their Use in Environmental Monitoring 74
3.1 Specific Soil Bioreporters 84
3.2 Bioreporters Specially Designed to Assess Bioremediation Programs and Wastewater Toxicity Evaluation 84
3.3 Freshwater Bioreporters 86
3.4 Eukaryotic Bioreporters 87
3.5 Aequorin-Based Bioreporters 88
4 Relevance of QSAR Models and Hormesis in Ecotoxicology 97
4.1 Bioluminescent Microorganisms and QSAR Models 97
4.2 Hormetic Response of Bioluminescent Organisms and Its Implications in Ecotoxicity Testing 101
5 Use of Bioluminescent Microorganisms to Assess Mixture Toxicity: From Whole Toxicity Determination to Development of Conceptual Models to Study the Toxicity of Complex Mixtures of Pollutants 103
5.1 Whole Toxicity Assessment 103
5.2 Bioluminescent Microorganisms and Toxicology of Mixtures 105
6 Concluding Remarks 114
References 115

1 Introduction

The contamination of natural environments is a worldwide problem that poses a danger to the health of humans and other organisms. Sophisticated chemical analysis techniques allow the quantification of the concentrations of a great number of contaminants with high accuracy and sensitivity, such as inductively coupled plasma, gas chromatography, and liquid chromatography mass spectrometry. These costly techniques usually require complex and analytical laboratory equipment, as well as pretreatment of the sample or extensive extraction from the environmental matrix. Most importantly, they do not allow the detection of pollutant bioavailability as the total pollution concentration does not usually correlate with the true impact of the pollutant to the biota. Chemical analyses do not respond to the important question: Is the sample toxic?

In this context, toxicity bioassays may complement chemical analyses and give information on the whole impact of the contaminated sample, which is a complex mixture of parameters such as bioavailability (the portion of the toxic substance that can be adsorbed/transported into an organism and potentially damage cell components), speciation (critical in the case of metals and metalloids), solubility (critical in the case of many organic pollutants), and potential toxicological interactions, such as synergism or antagonism when pollutants occur in complex mixtures, which is the most realistic scenario in natural environments. Because

the toxic impact may not be the same to humans, animals, plants, fungi, algae, and bacteria, there is a need to use a battery of bioassays with a variety of species from different trophic levels to realistically predict environmental hazards to the biota.

A large variety of toxicity bioassays have been developed, mostly based on higher organisms such as plants, algae, invertebrates, fishes, birds, and mammals (rodents). Many of these bioassays are standardized and validated by organizations such as the International Organization for Standardization (ISO), the American Society for Testing and Materials (ASTM), the Organization for Economic Cooperation and Development, the U.S. Environmental Protection Agency (US EPA), and Environment Canada. Most of these bioassays are based in the assessment of toxicity endpoints such as survival, growth, or reproduction; many are laborious and may take several days due to the slow response times. In addition, there might be ethical issues in the case of fish, birds, and mammals because animal sacrifice is usually involved [1, 2]. Therefore, there is a rising interest in developing cost-effective, less laborious, and rapid biological test systems [3].

The use of bioluminescent microorganisms has emerged as a powerful tool in environmental monitoring due to the ease of use, rapid response, and mass production. In many cases, immobilization/miniaturization allows the integration of the bioluminescent microorganism within optic transducers to form devices known as biosensors, which may be useful for continuous, online, and in situ monitoring of toxicity or stress, permitting potential rapid multitarget analysis [4–8].

Figure 1 summarizes the concept of the chapter, which includes a detailed description of the main characteristics and applications in ecotoxicity monitoring of pollutants of both naturally and recombinant (transgenic) bioluminescent microorganisms. The toxicity bioassays that use these bioluminescent microorganisms are based on the fact that light emission is reduced or even fully inhibited in the presence of toxic compounds that impair their metabolism.

2 Naturally Bioluminescent Microorganisms: Their Use in Environmental Monitoring

Bioluminescence is a natural phenomenon that has been observed in a variety of organisms, including insects, fish, jellyfish, clams, snails, crustaceans, fungi, algae, and bacteria [9, 10]. The reaction mechanisms of eukaryotic insect bioluminescence and bacterial bioluminescence are quite well understood. Bioluminescence is highly developed in insects. Three families belonging to the order Coleoptera—*Elatiridae* (click beetles), *Phangodidae* (the immature forms known as railroad worms), and *Lampyridae* (fireflies)—share the same substrate luciferin but naturally emit light of different wavelengths. Fireflies emit in the green-yellow region of the spectrum (maximum around 560 nm) and click beetles in the green-orange spectrum (540–613 nm); railroad worms span the widest range of the spectrum, from the green

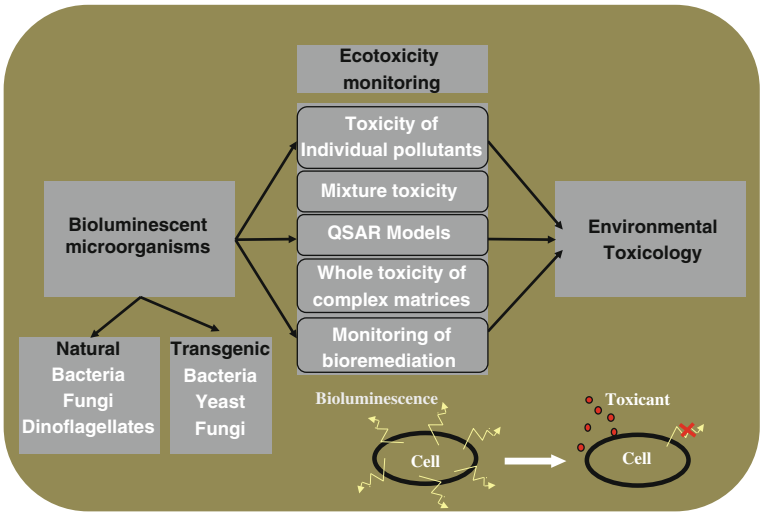

Fig. 1 Summary of bioluminescent microorganisms and their main applications in ecotoxicity testing of pollutants. The presence of a toxicant that impairs their metabolism causes inhibition of bioluminescence

(542–574 nm) to the red region (628–638 nm) [11–13]. The firefly (*Photinus pyralis*) bioluminescence system is the most studied and well characterized. The firefly luciferase encoded by the *luc* gene catalyzes the adenosine triphosphate-dependent decarboxylation of a luciferin in the presence of oxygen, which leads to the emission of light; however, the chemical origin of insect bioluminescence color modulations is still unclear [14]. The *luc* gene and mutant variants as well as the bioluminescent gene systems of click beetles and railroad worms offer the possibility of multicolor luciferase assays, which are used in many basic and applied research applications, such as reporter assays in vitro and in vivo [15] and in the construction of bioreporters for environmental monitoring [12, 14, 16].

Bioluminescent bacteria are light-emitting microorganisms found in marine, freshwater, and terrestrial ecosystems [10, 17, 18]. These bacteria are all Gram negative and include four genera: *Vibrio*, *Photobacterium*, *Shewanella,* and *Photorhabdus* (previously *Xenorhabdus*). *Vibrio*, *Photobacterium,* and *Shewanella* are found in marine environments while *Photorhabdus* is terrestrial. The bacterial luciferase, encoded by *luxAB*, involves the oxidation of $FMNH_2$ and a long-chain fatty aldehyde to produce the oxidized flavin (FMN) and a long-chain fatty acid with the emission of blue light (490 nm). The fatty aldehyde is synthesized by a reductase, a transferase, and a synthetase encoded, respectively, by *luxC*, *luxD,* and *luxE* [19–22].

Bioluminescence depends widely on cell metabolism because high-energy cofactors are required, so any toxic substance that may compromise the cell's metabolic status will cause a decrease in light emission proportional to the toxic

compound concentration. Luminescence is a noninvasive method easily detected with a simple photometer or photon-counting video cameras that are highly sensitive and allow continuous real-time monitoring; thus, the inhibition of bioluminescence by potential toxicants offers a sensitive, in vivo, and quick toxicity endpoint specifically targeting cell metabolism. For this reason, there has been a growing interest in using both eukaryotic and prokaryotic bioluminescence systems for environmental monitoring purposes [23].

The first natural luminescent microorganism to be used for assessment of environmental toxicity was an isolate of the marine bioluminescent bacterium *Vibrio fischeri* strain NRRL B-11177 (initially classified as *Photobacterium phosphoreum*) [24]. Although recently reclassified as *Aliivibrio fischeri* [25], most publications and applications, including commercial ones, still keep the term *V. fischeri*; therefore, for the convenience of the reader, *V. fischeri* will be used throughout this chapter, including the tables. The system was soon marketed as Microtox™ by Microbics Corporation (Carlsbad, CA, USA; later renamed as AZUR Environmental, http://www.azurenv.com. The system consists of freeze-dried bacteria that are reconstituted prior to the assay; their light production is measured by a dedicated luminometer (Microtox Analyzers) and specific software (MicrotoxOmni™). The measure of toxicity is given as the EC_{50}, the effective concentration of the substance that reduces light emission to 50 % of the original light emission.

Regulatory institutions and environment protection agencies only accept toxicity bioassays as analytical tools in environmental monitoring if enough standardization and validation has been performed. Hernando et al. [26] carried out an extensive research involving 10 European laboratories to validate the luminescence inhibition assay of *V. fischeri*; the assay proved to be satisfactory in terms of intra- and interlaboratory reproducibility and stability, and the use of different commercial tests did not make a significant difference. The procedure is fully standardized in the ISO11348 (http://www.iso.org/iso/catalogue_detail.htm?csnumber=40518) method, and it is an approved regulatory test in several countries, such as Canada, Australia, the United Kingdom, Sweden, The Netherlands, the United States (ASTM Standard D-5660-96; the US EPA has adopted Microtox™ as a standard test in an ongoing program of assessment and remediation of contaminated sediments), and Germany (German Standard Methods for the Examination of Water, Wastewater and Sludge-Bioassays [Group L]–Part 34. Determination of the inhibitory effect of wastewater on the light emission of *P. phosphoreum* [DIN 38412–part 34]). In China, a similar test to Microtox™ based on *V. fischeri* T3 sp. has been recommended by The Chinese Environmental Protection Agency for environmental analysis (China-NEPA 1995; Ma et al. [27]).

The Microtox™ method has been designed as an acute toxicity test, with incubation times ranging from 5 to 30 min; a long-term assay (24 h) has been proposed to assess the toxicity of specifically acting chemicals [28]. Besides Microtox™, other commercial devices based on the same principle, such as LUMISTox from Beckman Instruments and Tox-Alert from Merck, are available.

There is a large body of published data using Microtox™. An important fraction of such generated data is collected in the monographs by Kaiser and Palabrica [29] and Kaiser and Avillers [30]. Also, studies have shown that Microtox™ toxicity results correlate well with those from other toxicity bioassays such as fish, Daphnia, crustacean, ciliate, and algae [2]. The environmental applications of Microtox™ include testing of interstitial and municipal effluents, industrial process waters, hazardous waste, samples with biological toxins, contaminated soils and sediments, and assessment of the course of remediation/bioremediation processes [31–40]. Recently, *V. fischeri* bioluminescence tests have been used to assess the potential toxicity of engineered nanoparticles, which are considered as ultraemerging pollutants due to their increased production and subsequent release to the environment [41–52].

Deheyn et al. [53] used a wild-type strain of *P. phosphoreum,* originally collected from symbiotic bacterial light organs of the fish *Ophistoproctus soleatus,* to study the chemical speciation and toxicity of different metals by measuring the decrease in bioluminescence as a proxy for cell toxicity; this was compared to results obtained with Microtox™ and photocytes isolated from the brittle star *Ophiopsilla californica*. Hassan and Oh [54] also used a strain of *P. phosphoreum* denoted as KCTC 2852, which was obtained from the Korean-type culture collection, to detect toxic chemicals by measuring bioluminescence inhibition.

Girotti et al. [17] isolated the luminescent bacterial strain *Vibrio logei* from the Mediterranean Sea and used it to evaluate the toxicity of heavy metals, organics and a wide range of pesticides. The assay works at room temperature and can be measured in a simple nonthermostated microplate reader.

Ulitzur et al. [55] reported a new toxicity bioassay based on another natural bioluminescent marine bacterium: *Photobacterium leiognathi* strain TANI-1 isolated from the Red Sea. The bioassay has been marketed by Check-Light Ltd. (http://www.checklight.biz/; Israel) as the ToxScreen toxicity test. Like Microtox™, ToxScreen also uses freeze-dried bacteria. However, it is supplemented with two buffers: a pro-organic buffer that favors the detection of organic pollutants and a Pro-metal buffer that favors the detection of heavy metal cations. The test is sensitive after 30 min of exposure, although 60 min is recommended for maximal sensitivity. Results by Ulitzur et al. [55] indicated a greater sensitivity than that reported with the Microtox™ method. It has already been used in real natural samples, including river samples and heavy metal contaminated soils, with good results [56]. ChekLight Ltd. has launched a new ToxScreen test, ToxScreen II. This test is also based in a variant (strain SB) of *P. leiognathi,* which is more temperature tolerant than variant TANI-1 (ToxScreen I version) and may work in a wide range of temperatures (18–35 °C). The ToxScreen II test has recently been updated to ToxScreen-3 by the company as a kit for screening water from sources such as ground water, treated drinking water, surface water, and runoff water.

The above-mentioned luminous bacteria, although very useful, have a series of drawbacks. They usually operate in narrow pH and temperature ranges; for example, Microtox™ works in the 6.5–7.5 pH range and at 15–17 °C [24, 57]. The main disadvantage is due to the fact that they are marine organisms and NaCl

has to be added to every sample to reach a final concentration of 2–3 % salt; this high salt concentration has been found to influence the speciation and subsequent toxicity of metals [58–60] and may alter the solubility of some organic substances producing turbid solutions [1]. Thus, the inherent properties of freshwater samples may be altered when these marine bacteria tests are used. There is increasing interest in finding freshwater luminous bacteria for this type of sample. Ma et al. [27] isolated the freshwater luminescent bacterium *Vibrio qinhaiensis* sp.-Q67 from the body surface of the edible fish *Cymnocyprus przewalskii,* which exhibits a wide pH tolerance. They tested this strain with several toxicants (metals and pesticides) and compared it to the performance of the marine *V. fischeri* strain T3, finding that it is as effective and reliable as conventional *V. fischeri* assays. The use of *V. qinhaiensis* sp.-Q67 is expanding and numerous studies report its use with a wide variety of pollutants, mixtures of pollutants, and environmental samples [61–71].

Bioluminescent bacteria are widely used in environmental monitoring. However, there are criticisms about their usefulness for estimating the effect of pollutants on eukaryotic organisms. For this reason, there is an increasing number of reports on the development and application of biotests with higher organisms that are also naturally luminescent, such as fungi and dinoflagellates.

Bioluminescent fungi are claimed to be useful, specifically for terrestrial environments [72–75]. Approximately 80 species of luminous fungi have been reported and new species are being continuously discovered [72, 76, 77]. All of them are basidiomycetes and belong to at least three distinct evolutionary lineages: *Omphalotus, Armillaria,* and *mycenoid* [76]. In most of these basidiomycetes, the globular mycelium (a complex network of microscopic filamentous hyphae) is luminescent. Cultures of these mycelia both in liquid and agar have been used for environmental monitoring of different heavy metals, organics, and terrestrial environmental samples [72, 73, 75, 78]. As in bacteria, the bioassays are based on the decrease of the intensity of bioluminescence when exposed to increasing concentrations of the potential toxic compound; all luminous fungi emit blue-green light with a maximum at 520–530 nm [79]. The influence of culture conditions on growth and bioluminescence of mycelia growth of different fungi have been reported by Weitz et al. [80] and Mendes et al. [72]. Fungi have been reported to be luminescent in a wide temperature range, from 4 to 50 °C [77]. Exposure times to pollutants between 30 min and 24 h have been reported as necessary, depending on the species and whether liquid or agar-mycelia cultures are used [72, 73, 75, 77]. Depending on the species, the pH range of bioluminescence is between 3 and 6 [73, 75].

With these biotests, the metabolism of the fungi has been targeted; however, currently the mechanisms of fungi bioluminescence are not as well understood as those in bacteria [76, 77]. Some researchers have proposed an enzymatic mechanism involving a NADPH-reductase and a luciferase, which might oxidize a putative fungal luciferin resembling the mechanism of bacterial bioluminescence [81–83]. However, the chemical structure of the luciferin is unclear and the enzymes have not been purified [76]. There is even a proposal [84–86] that

suggests a nonenzymatic mechanism for fungal bioluminescence; if that is the case, the use of fungal bioluminescence as a toxicity bioassay targeting metabolism can no longer be considered. Nevertheless, efforts are underway to try to isolate putative luciferin and enzymes involved in fungal luminescence [76].

Dinoflagellates are protists, which are common organisms in any type of aquatic ecosystem, marine or freshwater. They can be autotrophic or heterotrophic, being relevant components of phytoplankton and zooplankton. Autotrophic dinoflagellates play an important role as primary producers in marine environments; thus, they are ecologically relevant as test organisms to evaluate the toxicity and bioavailability of pollutants in oceans. Some marine autotrophic dinoflagellates are able of producing bioluminescence when exposed to different types of stimuli, emitting light in the range of 470–490 nm. It has been proposed that light emission in dinoflagellates acts as an indicator of the presence of grazers to higher trophic levels [87].

Lapota et al. [88] were the first to develop a toxicity bioassay based on bioluminescent dinoflagellates to evaluate acute and sublethal toxic effects. Being marine organisms, the test needs salt in the assay media and should be more appropriate for marine environments. These researchers have already developed a commercial test denoted as QwikLite [89–91], which is marketed by Assure Controls Inc. (www.assurecontrols.com; California, USA) as a test to assess toxicity of effluents, industrial discharges from naval facilities, antifoulant paints, bay sediments, elutriates, and sediment interstitial waters. The test is based, as other bioluminescent assays, in the decrease of dinoflagellate luminescence following 24 h exposure to the potential toxic sample. The most used dinoflagellate species in the QwikLite test are *Lingulodinium polyedrum*, *Ceratocorys horrida,* and *Pyrocystis noctaluca,* which are cosmopolitan and easy to culture and maintain [90]. Besides QwikLite, the test LUMITOX$^{(R)}$ (Lumitox Gulf L.C. River, Ridge, LA, USA) has been developed to measure quenching, in the presence of toxins, of the natural bioluminescence of the abundant marine dinoflagellate *Pyrocystis lunula* (U.S. Patent #4,950,594-1990) [92, 93]. The test uses a bioluminescence-measuring device (Tox-Box). Procedures based on LUMITOX® and QuikLite were developed into ASTM method ASTM E1924-97, which was reapproved in 2004 and 2012; however, the latter was withdrawn in 2013 without replacement due to its limited use by industry (www.Astm.org/standards/E1924.htm).

Bioluminescent dinoflagellate bioassays have been used to assess the toxicity of some heavy metals, organics such as polycyclic aromatic hydrocarbons, and sediments polluted with metals and organics [89, 90, 92, 94, 95]. Some researchers have reported that dinoflagellate luminescence inhibition may be a more sensitive toxicity endpoint than *V. fischeri* luminescent inhibition [90, 96].

In most naturally luminescent organisms, bioluminescence levels may oscillate depending on growth conditions, stage of growth, cell concentration, sufficient concentration of autoinducer in bacteria (a small organic molecule required to maintain bioluminescence in culture), supply of intracellular reducing power for luciferase, or dissolved oxygen concentration [97–100]. For optimization of light

emission, the choice of growth medium and culture conditions is of outmost importance. In this respect, Scheerer et al. [18] optimized the conditions for continuous cultivation of *V. fischeri* NRRL-B-11177 in a fermenter, providing a reliable long-term (more than 1 month) continuous culture method that allowed the reproducible measurement of changes in the bacterium metabolism by monitoring changes in bioluminescence.

Table 1 provides a summary of naturally luminescent microorganisms, featuring the species, type of organism, origin, commercial devices if available, and main applications.

3 Recombinant Bioluminescent Microorganisms: Their Use in Environmental Monitoring

As discussed in the previous section, Microtox™ and other proprietary assays using *V. fischeri* have been widely used in ecotoxicity evaluation of pollutants in marine and terrestrial environments, but their use in terrestrial environments (soils, sediments, and freshwaters) has several limitations. The main limitation is due to the need for high salt concentration in the assay to achieve optimal bioluminescence. As discussed above, this salt concentration can alter the solubility and/or bioavailability of compounds in environmental samples. Other limitations are that they only function in a limited range of pH and temperature. Another important disadvantage is that *V. fischeri*, a marine organism, is not representative of terrestrial ecosystems and may respond in a different way to toxicants than more representative microorganisms. Finally, other limitations come from the need for more resistant, less sensitive organisms if the ecotoxicity evaluation is going to be performed in wastewater or contaminated soils; on the contrary, there is also a need for more sensitive organisms if the assay is to evaluate unpolluted freshwaters or ground water. For these reasons, transgenic bioluminescent microorganisms have been developed to meet the needs of assessment of different terrestrial (soil and freshwater) environments. These transgenic organisms harbor luminescent genes from bioluminescent bacteria (*lux* genes from *V. fischeri, V. harveyi* or *P. luminescens*) or firefly (*luc* from *P. pyralis*) coupled to constitutive promoters to allow continuous expression of the bioluminescent genes; the luminescence of these transgenic microorganisms, as in the case of the naturally bioluminescent organisms, is turned off in response to toxic compounds present in the environment. They are usually named lights-off or metabolic bioreporters [4–8]. In addition, *lux* and *luc*-based bioreporters are transgenic microorganisms expressing the apoaequorin gene, which targets intracellular calcium homeostasis; they have been used to assess environmental toxicity and will be also discussed in this section.

Although *Escherichia coli* is not an ecologically relevant microorganism, bioluminescent transgenic strains have been successfully used in evaluating inland waters, soil, and air ecotoxicity due to their ease of use, knowledge of the genetics,

Table 1 Main features and applications of naturally bioluminescent microorganisms

Organism	Organism type/Origin	Commercial tests	Applications	References
V. fischeri (several strains)	Prokaryotic/Seawater	Microtox™ LUMISTox® Tox-Alert®	Evaluation of the toxicity of thousands of compound applied both singly and in mixtures, natural samples, and monitoring of remediation processes	[24, 29, 30, 32, 38]
P. phosphoreum (several strains)	Prokaryotic/Seawater		Testing of the speciation and toxicity of heavy metals	[53, 54]
V. logei	Prokaryotic/Seawater		Evaluation of the toxicity of heavy metals, organics, and wide range of pesticides	[17]
P. leiognathi	Prokaryotic/Seawater	ToxScreen®	Evaluation of the toxicity of heavy metals and organics; wide range of environmental samples	[55, 56]
Vibrio-qinghaiensis sp.-Q67	Prokaryotic/Freshwater		Evaluation of the toxicity of a wide variety of pollutants applied both singly and in mixtures; testing of environmental samples	[27, 62–64, 66, 67, 69, 71, 349]
A. mellea, *A. Borealis*, *A. gallica*	Eukaryotic (Fungi)/Soil		Evaluation of the toxicity of the toxicity of heavy metals, organics, and environmental samples	[73, 77, 80]
Mycena citricolor (ATCC34884)	Eukaryotic (Fungi)/Soil		Evaluation of the toxicity of heavy metals	[75]
Gerronema viridilucens CCB691	Eukaryotic (Fungi)/Soil		Evaluation of the toxicity of heavy metals	[72]
Lampteromyces japonicus	Eukaryotic (Fungi)/Soil		Evaluation of the toxicity of heavy metals and organics	[77]
L. polyedrum *C. horrida* *P. noctaluca*	Eukaryotic (Dinoflagelate)/Seawater	QwikLite®	Evaluation of the toxicity of heavy metals, organics, and marine environmental samples	[90, 91, 94]
Pyrocystis linula	Eukaryotic (Dinoflagellate)/Seawater	LUMITOX®	Evaluation of the toxicity of heavy metals, organics, and marine environmental samples	[92, 93]

and the availability of numerous strains that can be tailored to the needs of environmental bioassays. They have been used alone or in combination with other ecotoxicity assays involving other organisms, some of them also bioluminescent (see below). A relevant recombinant strain of *E. coli* is being extensively used for environmental monitoring—*E. coli* HB101 (pUCD607), which harbors plasmid pUCD607 with *luxCDABE* from *V. fischeri* fused to the *tet* (tetracycline) promoter, providing constitutive luminescence expression [101].

This section first presents results on *E. coli* and other bacteria transformed with pUCD607, as well as other strains marked with *lux* genes from *V. fischeri*. Then, bacterial strains marked with *P. luminescens lux* genes are discussed.

E. coli HB101 (pUCD607) was originally designed for in situ detection of *E. coli* in soil. Later, it was used mainly in acute toxicity assays in soils contaminated and/or spiked with heavy metals [102–111]. The assays with luminescent bacteria were made in aqueous solutions with resuscitated lyophilized bacteria and aqueous extracts from the soils. The metal concentration in pore water is always several orders of magnitude lower than in bulk soil because metal can be complexed with soil components. The percentage of metal (Cu and Ni) soluble and free ion in amended soils depends mainly on the pH, the organic matter content, the texture, and the presence of Ca^{2+} and Mg^{2+} exchangeable cations on the soil [105, 112]. Zampela and Andano [110] reported higher soluble Zn and higher toxicity to *E. coli* HB101 (pUCD607) in acidic podozolic soils than in volcanic subalkaline spiked soils.

Other bacteria transformed with the plasmid pUCD607 and frequently used in combination with *E. coli* HB101 (pUCD607) are *Pseudomonas fluorescens* 10586 [102], a ubiquitous soil Gram-negative bacterium that constitutes the most abundant Gram-negative bacterial group in the rizhosphere [113]; *P. putida* F1, which is capable of degrading toluene [114]; and *Rhizobium leguminosarum* biovar *trifolii* [115, 116], a plant symbiont that supplies a large amount of fixed N_2 to clovers and is a sensitive indicator of soil pollutants commonly used in toxicity assays of nodulation and growth inhibition [116].

P. fluorescens 10586 (pUCD607) has been used to assess the effluent ecotoxicity of a malt whisky distillery, demonstrating sensitivity to Cu [117], and of a papermill treatment plant, whose principal contaminants were Cd and pentachlorophenol (PCP) [118]. *R. leguminosarum* bv *trifolii* F6 (pUCD607), in conjunction with *P. fluorescens* 10586 (pUCD607), has been used to assess the toxicity of arable soils fertilized with paper mill sludge. These soils showed reduced crop yields; water extracts from them reduced the luminescence of both bioreporters; the main contaminants of these soils were Cd, Cu, and PCP, but the toxicity to both bioreporters seemed to be due to PCP or to synergistic interactions because the two metal concentrations seemed to be lower than those detected by the bioreporters. *R. leguminosarum* bv *trifolii* F6 (pUCD607) was especially sensitive to Cd, whereas *P. fluorescens* 10586 (pUCD607) was more sensitive to Zn, Cu, and Ni [116]. Both bioreporters showed more sensitivity in a chronic assay (72 h of exposure) than in an acute assay (30 min of exposure) [116].

MacGrath et al. [112] reported higher soluble Zn and free ion in a forest soil, with lower pH, than in an arable soil amended with sewage sludge; they reported higher toxicity to *P. fluorescens* 10586 (pUCD607) than to *E. coli* HB101 (pUCD607). Paton et al. [119] reported in soils contaminated by Ni and Cu from smelters a good correlation between soluble Cu and inhibition of luminescence in *E. coli* HB101 (pUCD607) and *P. fluorescens* 10586 (pUCD607), but no correlation with soluble Ni was found. In the same study, a series of distinct ecotoxicity assays were made showing that minimal inhibitory concentration for heterotrophic microbial growth correlated well with soluble Ni and Cu except in the more contaminated soils; an assay with a natural luminescent fungus (*Armillaria mellea*) was less sensitive and reproducible. From this work, they concluded that it is important to use a battery of different toxicity assays with organisms covering a wide range of trophic levels.

When comparing the sensitivity of *E. coli* HB101 (pUCD607) and *P. fluorescens* 10586 (pUCD607), Chaudri et al. [102] found that *E. coli* was more sensitive to Zn than *P. fluorescens* but did not respond to Cu, probably because the free ion Cu^{2+} was very low, under the detection limit. Vulkan et al. [109] assayed contaminated soils from the UK, Chile, and China, showing that *E. coli* responded similarly to soluble Cu and free ion Cu^{2+} but *P. fluorescens* luminescence response correlated better with Cu^{2+}. Flynn et al. [104] also showed that *P. fluorescens* is less sensitive to Cu and As from soils from a mine zone in Antofagasta (Chile). However, Dawson et al. [120] did not find significant differences between *E. coli* HB101 and *P. fluorescens,* but both were significantly more sensitive than the soil transgenic bioluminescent bioreporter, *R. leguminosarum* bv *trifolii* TA1-Tn5*luxAB* (see below).

E. coli HB101 (pUCD607) also was used to detect acute toxicity of aqueous solution of Zn, Cu, and Cd mixtures and their possible interactions, synergism, or antagonism [121], and also of soils spiked with metal mixtures [107]. Interaction of Zn, Cu, and Cd with ethylenediaminetetraacetic acid and fulvic acids (chelating acids normally present in soils) showed that there is not always a simple relationship between free-ion metal and toxicity, but the relationship is higher than with total metal concentration [122].

Besides its use in heavy metal toxicity, *E. coli* HB101 (pUCD607) has been used in toxicity determination of organic contaminants in soil and freshwater. Trott et al. [108] made a comprehensive study comparing the performance of *V. fischeri*, *E. coli* HB101 (pUCD607), and *P. fluorescens* 10586 (pUCD607) with other bioassays in testing the ecotoxicity of aqueous or soil extracts amended with heavy metals (Zn and Cu), and organic contaminants (chlorophenols, herbicides, organotins, polycyclic aromatic hydrocarbons [PAHs], and refined hydrocarbons). In aqueous solutions, *E. coli* and *P. fluorescens* were more sensitive to Cu and Zn than *V. fischeri*. In soil solutions, heavy metal toxicity measured by *E. coli* gave a nonlinear correlation with the earthworm accumulation assay. The responses of bacterial luminescent bioreporters to organic contaminant from soils varied depending on the extraction method. Chlorophenols were more toxic in aqueous extracts than in methanol extracts, with *E. coli* and *P. fluorescens* being always

more sensitive than *V. fischeri*. Sensitivity to herbicides depended on the herbicide, being always more toxic in methanol extracts. *V. fischeri* was more sensitive to organotins, but toxicity only appeared in methanol extracts. None of the strains responded to PAHs other than naphtalene or refined hydrocarbons in aqueous solution, and the response was very poor in methanol extracts. In historically contaminated soils, there was no correlation between toxicity measured by the three luminescent bacteria and the extracted amount of hydrocarbons. Reid et al. [123] also found that *E. coli* HB101 (pUCD607), *P. fluorescens* 10586 (pUCD607), *V. fischeri, and P. putida* F1 (pUCD607) luminescent strains were not responsive to PAHs in aqueous solution; only *R. leguminosarum* biovar *trifolii* (pUCD607) showed decreased luminescence in the presence of PAHs. However, Bundy et al. [124] used *E. coli* (pUCD607), *P. putida* F1 (pUCD607), and *V. fischeri* together with three lights-on bioreporters to monitor the bioremediation of five oils in soils, finding an increase of toxicity along the experiment (119 days) as reported by the three metabolic bioreporters. Dawson et al. [125] also used *E. coli* HB101 (pUCD607) and *P. fluorescens* 10586 (pUCD607) in conjunction with other ecotoxicity assays to assess remediation of hydrocarbon-contaminated soils, concluding that both bioluminescent bioreporters were inadequate for inclusion in the soil quality index because they could not discriminate between soils.

Toxicity of organotins, such as triphenyltin (TPT, used as fungicide in crops) and tributyltin (TBT, antifouling agent) was assessed with *E. coli* HB101 (pUCD607), *P. fluorescens* 10586 (pUCD607), and *V. fischeri* by Paton et al. [106], who showed that in aqueous solutions *V. fischeri* was the more sensitive bioreporter (as also reported by Trott et al. [108] in soil) and *E. coli* was the less sensitive. In soil extracts, the concentration of these organotins needed for toxicity was higher than in aqueous solutions and depended on the pH of the soil. TPT degradation occurred in soils both biotically (with 27–33 days of half-life) and abiotically. When toxicity during TPT degradation was assessed by *P. fluorescens* 10586 (pUCD607), there was always an enhancement of toxicity the first days, indicating that intermediary metabolites were more bioavailable and/or toxic; afterwards, toxicity decreased. Previously, Bundy et al. [126] described that the primary products of degradation of TBT and TPT, triphenyltin and diphenyltin, respectively, were more toxic to *P. fluorescens* 10586 (pUCD607) than their precursors and, curiously, soil extracts of organotins were one order of magnitude more toxic to *V. fischeri* than aqueous solutions of those compounds.

Chlorophenols are contaminants derived from herbicides, fungicides, general biocides, preservatives of wood, textiles and leather, pulp mill effluents, and disinfection of water by chlorine [127, 128]. In aqueous solutions, 2,4-dichlorophenol (DCP) was toxic to bioluminescent bacterial bioreporters, with Microtox™ (*V. fischeri*) being more sensitive than *E. coli* (pUCD607) and *E. coli* more sensitive than *P. fluorescens* (pUCD607). Lower pH levels increased the toxicity to *E. coli*, corresponding with higher adsorption to the bacterial cell wall [129]. Later, Tiensing et al. [130] studied the interactions between DCP, trichlorophenol (TCP), and pentachlorophenol (PCP) in aqueous solutions and in two types of soils (Bondye and Insch), being synergistic to *P. fluorescens* and antagonistic to *E. coli* in

Bondye soil and turning into additive at low concentrations in Insch soil. Later, *E. coli* HB101 (pUCD607) proved to be useful in assessing remediation of DCP by photoelectrocatalysis in water [131] and by biodegradation with the bacteria *Burkholderia* sp. RASC c2 in soil [132]. However, Puglisi et al. [133] reported increasing toxicity to *P. fluorescens* 10586 (pUCD607) during degradation of PCP in compost-amended soils, suggesting the formation of more toxic metabolites or a synergistic effect between these metabolites, as highlighted by Tiensing et al. [130].

The ecotoxiciy of seven herbicides was tested in freshwater with *E. coli*, *P. fluorescens*, *P. putida* harbouring the multicopy plasmid pUCD607, and *R. leguminosarum* biovar *trifolii* TA1 *luxAB*. The *E. coli* bioreporter was the most sensitive and the *Rhizobium* bioreporter was the least sensitive. The toxic effect of two herbicide mixtures to the *E. coli*-based bioreporter depended on the herbicides and on their concentration in the mixtures [134]. When the ecotoxicity *to E. coli* pUCD607 bioreporter was measured in water extracts from two spiked soils with four herbicides [135], it was shown that the concentration necessary to exert toxicity was lower for all the herbicides than the concentration needed in aqueous solutions, indicating an interaction with soil components which made herbicides more toxic.

E. coli HB101 (pUCD607) was also used to assess the toxicity and remediation of groundwater boreholes contaminated with chlorinated aliphatic hydrocarbons (CAHs) in combination with *V. fischeri* and *P. fluorescens* 10568 (pUCD607). Two lights-on bioreporters—one responsive to alkanes and the other to simple aromatics and to some CAHs—were also used [136]. Chemical analysis identified the contamination of borehole samples as trichloroethene and trichloroethane. The most sensitive bioreporter was *V. fischeri* and the least sensitive was *E. coli* HB101 (pUCD607). *P. fluorescens* 10568 (pUCD607) and the two lights-on bioreporters showed an intermediate sensitivity. Two remediation strategies were used for remediation of water samples: air sparging and adsorption to activated charcoal. For all the bioreporters but *V. fischeri,* air sparging was enough to remediate the contamination, but the toxicity results obtained with *V. fischeri* indicated that activated charcoal was necessary to eliminate all the contamination. *P. fluorescens* is a CAH degrader, so it also gave information about the possibility of survival of a degrader community after bioremediation strategies.

An important technical advance using *E. coli* HB101 (pUCD607) is online biosensing using a device that allows one to directly quantify the toxicity over time with resuscitated freeze-dried cells [137] or with them immobilized in a polyvinyl alcohol matrix [138]. This toxicity fingerprinting—a "combination of dose response data with temporal response data"—allows one to make a three-dimensional map of known contaminants. With the aid of an algorithm and a calibration database, it also allows one to predict the contaminants present in an unknown sample. Turner et al. [137] reported that 83 % of natural spiked water samples were correctly detected at the 95 % test level using this experimental approach. The samples were better detected if the metabolite concentration was in the intermediate dose level of the bioreporter. Thus, using this technology, a lights-off bioreporter can provide qualitative and quantitative information, although it

may not function in a complex mixture. Horsburgh et al. [138] improved the method. Using *E. coli* HB101 (pUCD607) as a bioreporter, they predicted that the main toxicant of an effluent from a metal-plating plant was Zn; an organic contaminant similar to DCP as the main contaminant in an effluent from a paper mill (further chemical analysis identified PCP as the principal contaminant); and Cu mixed with an organic compound at low concentration (inductively coupled plasma–mass spectrometry identified Cu as the main contaminant) in an effluent from a distillery.

R. leguminosarum biovar *trifolii* TA1-Tn5luxAB [115, 116] and *P. fluorescens* 10586/FAC510 [139–141] are strains marked with *luxAB* genes from *V. fischeri* integrated in the chromosome. These strains do not harbor the *luxCDE* genes necessary for the synthesis of aldehyde; thus, it is necessary to add it exogenously. Probably for this reason, they are less sensitive than the above-mentioned strains transformed with plasmid pUCD607; in this regard, other strains such as *P. fluorescens* 8866 Tn5 *lux*CDABE, and *P. putida* F1 Tn5 *lux*CDABE with *luxCDABE* from *V. fischeri* have similar sensitivity than those transformed with pUCD607 [142]. *P. putida* F1 Tn5 *lux*CDABE has been used to monitor the degradation and toxicity of benzene, toluene, ethylbenzene and xylene (BETEX) compounds in soils in combination with a lights-on bioreporter (as opposed to lights-off bioreporters, the luminescence of these strains is induced in the presence of certain pollutants), proving that toxicity increased during the first days of the remediation process (probably due to the formation of more toxic compounds), decreasing afterwards [143].

P. luminescens luciferase has the advantage over *V. fischeri* luciferase that it is thermostable. *P. luminescens* was isolated from a human wound. Its optimum growth and luminescence temperature is 33 °C and the optimum temperature activity for the purified luciferase is 40 °C [144]. The optimum temperature for *V. fischeri* luciferase is 15 °C and is not stable at temperatures above 30 °C [145]. These temperatures are not generally compatible with conventional plate luminometers, and *V. fischeri* luciferase is not suitable to construct recombinant organism with optimum temperatures above 30 °C [146]. For these reasons, a series of transgenic microorganisms constitutively expressing *luxCDABE* from *P. luminescens* have been developed: *E. coli* MC106 (pDNlux), *E. coli* MC106 (pSLlux), *P. fluorescens* OS8 (pDNlux), *P. fluorescens* OS8::Knlux, *Bacillus subtilis* BR151 (pBL1/p602/22lux); *Staphylococcus aureus* RN44220 (p602/22lux) [147, 148]; and the cyanobacterium *Anabaena* CPB4337, which will be described later in this section under freshwater bioreporters [149].

pDNlux and pSLlux plasmids differ in copy number and in constitutive promoter. pDNlux is a medium copy number plasmid and luminescence is directed by the T7 promoter, whereas pSLlux is a high copy number plasmid and luminescence is directed by the *lac* promoter. *P. fluorescens* OS8 is a nonpathogenic soil bacterium isolated from a fungal hyphae of toluate-contaminated soil [150]; it has been transformed with pDNlux plasmid or bears the *lux* operon inserted in the genome and fused to the T7 promoter (*P. fluorescens* OS8::Knlux) [147]. *B. subtilis* and *S. aureus* are

Gram-positive bacteria; they have been transformed with a plasmid where the *lux* operon is fused to the *lac* promoter. Although *B. subtilis* is a spore-forming bacterium common in soils, *S. aureus* is not ecologically relevant but is medically important being an opportunistic human pathogen. These bioreporters have been mainly used for control of the performance of lights-on bioreporters based in the same strains to test for potential quenching of luminescence by sample turbidity or toxic effects. They also have been used as correction factors in studies of contamination by phenol in groundwater and semi-coke leachates [148]; Hg in soils and sediments form Aznalcollar (Spain) mining area using optic-fiber immobilized or nonimmobilized biosensors [151]; heavy metals and organic compounds in non-natural samples [146, 147]; oxidative stress by nanoparticles [152]; and effects of rhamnolipids on calcium bioavailability [153]. An important feature of these studies involving toxicity in soil samples is that toxicity assays with bioluminescent bacteria were made not in aqueous soil extracts but in direct contact with the soil. Previously, Ivask et al. [154] have shown, using the lights-on *B. subtilis* BR151 (pTOO24) and *S. aureus* RN44220 (pTOO24) bioreporters and their respective constitutive bioluminescent strains *B. subtilis* BR151 (pCS5962/pBL1) and *S. aureous* RN44220 (pTOO02) marked with firefly luciferase (*luc*) from *P. pyralis*, that Cd was 115-fold and Pb 40-fold more bioavailable in direct soil-bacteria contact than in aqueous soil extracts, with metal bioavailability changing depending on soil type.

Furthermore, the suitability of these *P. luminescens* luciferase marked-bioreporters as overall toxicity lights-off bioassays has been tested and compared with *V. fischeri*-based tests [146, 147, 153]. The main conclusions of these works was that there was higher luminescence with higher copy number, but the sensitivity was similar independently of the copy number. Gram-positive bacteria presented lower luminescence but higher sensitivity to heavy metals (one order of magnitude); the Gram-negative *E. coli* and *P. fluorescens* showed similar sensitivity to Hg, methylmercury, Pb, and Ag; but *E. coli* showed higher sensitivity to Zn (3-fold), Cu (15-fold), and Cd (100-fold) [147]. When comparing toxicity of heavy metals and organic compounds to *E. coli* and *V. fischeri*, the EC_{50} values from most of the chemicals were statistically different, but there was a good correlation between the EC_{50s} as logarithmic values from both organisms [146]. These studies also showed that the medium composition had significant effects on the toxicity to the *E. coli* strain: a M9 medium supplemented with glucose and other amino acids instead of leucine reduced the apparent toxicity of heavy metals up to three orders of magnitude, but had no effect on the organic compounds tested (aniline, 3,5-dichoroaniline and 3,5-dichlorophenol).

Rhamnolipids based-biosurfactants produced by *P. aeruginosa* are used in environmental remediation because they enhance the water extracted Cd binding to it, making it not bioavailable [155]. When comparing the toxicity of Cd *to E. coli*, *P. fluorescens*, *B. subtilis,* and *V. fischeri* bioluminescent strains in the presence of these biosurfactants, it was shown that these compounds reduced Cd toxicity to Gram-negative bacteria but not to the Gram-positive *B. subtilis*, indicating that rhamnolipids had effects not only by Cd complexation but also by inducing changes in the cell membranes.

Apart from *B. subtilis* and *S. aureus*, the only metabolic luminescent transgenic Gram-positive bacterium used in ecotoxicity assays is *Streptomyces lividans* (pESK004), which harbors the *luxAB* genes from *Vibrio harveyi* expressed constitutively in the plasmid pESK004. It is highly sensitive to heavy metals and herbicides and not sensitive to chlorophenols both in solution and in natural sample waters [156].

Another bioreporter constitutively expressing *luxCDABE* from *P. luminescens* is *E. coli* RFM433 GC2 [157]. It harbors the plasmid pLITE2 that contains *luxCDABE* fused to *lac* promoter [158]. It has been immobilized in a device constituting a biosensor. First, it was immobilized in a LB-agar matrix deposited in polypropylene tubes of 7.5 mm in diameter and 10 mm in length, with one end connected to a luminometer through optic fiber constituting a disposable biosensor kit. The incubation with the sample was performed in a two-test chamber coated with a thermostatic water jacket to maintain the optimum temperature, a disposable biosensor kit was inserted in each test chamber, and the sample was introduced in one test chamber; in the other, a control sample (nontoxic) was introduced. The luminometers were connected to a computer to obtain a continuous reading. This format, with a 100-ml chamber, was successfully used to detect benzene in air samples [157]. Later, the method was improved by two means. First, the bioreporter cells were immobilized in 20-mm diameter, 32-mm length glass tubes. Second, 425- to 600-mm glass beads were included in the LB-agar matrix to immobilize the bioreporter cells. These changes improved the sensitivity to toluene from 48 to 15 ppm due to enhancing the gas diffusion through the matrix [159]. In this study, the toxic volatile BETEX were also tested. This biosensor is the only one based on a lights-off recombinant microorganism suitable for detecting gas toxicity in air samples.

A similar biosensor format with the same strain, but with a 50-ml chamber and the biosensor kit submerged in the solution test that was air bubbled, was developed to measure toxicity in aqueous samples. In this case, the biosensor kit was immobilized in 7.5-mm diameter, 10-mm length polypropylene tubes with 0.05 g of glass beads; it was used to measure PAHs in soil pore water extracted with rhamnolipids [160]. The small size of the biosensor makes it useful to monitor toxicity in field samples in situ. Choi and Gu [161] developed a smaller portable toxicity biosensor; in this case, the cells were freeze-dried in 1.5-ml glass vials. The vial was connected to an optic fiber and then introduced into a small biosensing chamber; there, the cells were rehydrated by injecting water. After 30 min of acclimation, the toxic sample was added. This format has been used to test phenol and chlorophenols compounds using the *E. coli* RFM433 GC2 strain and several other lights-off recombinant luminescent strains [161].

The *E. coli* RFM433 GC2 strain was also used in a nonimmobilized format to test the toxicity of Azo dyes [162] and gamma-radiation [163], responding in a dose-depending manner. Furthermore, it was used in a multichannel system for continuous monitoring [164–167]. Each channel consisted of two minibioreactors—one (20 ml) to grow the recombinant bioluminescent strain, another (10 ml) to mix the recombinant strain with the sample solution, both connected by optic

fiber to luminometers and to a computer. Each channel harbored a different lights-on bioreporter, but one channel harbored the general toxicity (lights-off) bioreporter (*E. coli* RFM433 GC2) [164]. This system permits one to classify the toxicity of the sample [164]. It has been used to test the toxicity of the coolant water from a nuclear and a thermoelectronic power plant in Korea [165], as well as, in a continuous manner, the effluents of a wastewater treatment plant and two rivers in Germany [166].

Another way to detect toxicity with these bioreporters is the use of cell arrays in microchips or in plates. In the first case, the chip is immersed in solution with the toxicant; then the luminescence measured with a highly sensitive cooled charge-coupled device (CCD) camera. In the second case, the toxicant is added to each well of the plate and the luminescence measured either with a CCD camera or with a luminometer (reviewed in [168]). *E. coli* RFM433 GC2 has been included in cell arrays as a general toxicity strain in combination with several lights-on strains immobilized either in LB agar [169–171] or sol-gel [171]. In general, the response of the strains was similar in sol-gel and LB-agar and they were less sensitive than when nonimmobilized, probably due to the fact that the immobilization matrix may affect the sensitivity to several chemicals by interactions with them. Furthermore, the luminescence of all the strains (particularly GC2) is more stable if stored immobilized in the LB-agar matrix [171]. It was, however, difficult to detect changes in luminescence for GC2 strain when using a microchip and a CCD camera [169]. When comparing GC2 and lights-on strains performances in cell arrays or a multi-channel system, normally the sensitivity of GC2 was lower to all tested toxicants that were specific to the lights-on strain, and some toxicants did not inhibit its luminescence. This is because the inducible promoters present in the lights-on bioreporters were expressed early after contact with the toxicant inducing mechanisms to mitigate damage. At this stage, lights-on bioreporter luminescence increased in a dose-dependent manner to a maximum level. However, at high toxicant concentrations, toxicity was observed as lights-off bioreporter luminescence decreased, and no further increases in luminescence were detected in the lights-on bioreporters [172].

Salmonella typhimurium is a nonecologically relevant bacterium, but traditionally it has been used for the Ames test, which detects genotoxicity. The VITOTOX® test is a variant of the Ames test that uses bioluminescence in which the *V. fischeri lux* operon is expressed under the *recN* promoter (one of the genes that belongs to the SOS system, which is activated to repair DNA damage); it is expressed in the presence of genotoxic compounds. To normalize the response of this strain with respect to possible cytotoxic effects, an improvement was made by including in the assay another *S. typhimurium* strain called TApr1, which harbors the *lux* operon under a constitutive promoter selected by random cloning of EcoR1 digested fragments from *Alcaligenes eutrophus* CH34, a soil Gram-negative bacterium isolated from a site polluted by heavy metals. The inclusion of this lights-off strain in the VITOTOX® test highly improved the reproducibility of assays between different laboratories [173].

3.1 Specific Soil Bioreporters

So far, we have reported recombinant bioluminescent bioreporters used to detect toxicity in several matrices, including aqueous solutions, freshwaters, soils, sediments, and gases, although some of them were initially designed to detect toxicity in soils. Now, we shall focus in two bioreporters based on bacteria isolated from soils and used exclusively to detect toxicity in soils: *Nitrosomonas europaea* ATCC 19781 (pHLUX20) and *P. fluorescens* DF57-40E7. Both carry the *luxAB* genes—the first one in a plasmid and the second one integrated into the genome.

N. europaea is an autotrophic ammonia-oxidizing soil bacterium responsible for the initial rate-limiting step of nitrification in soils. It plays a key role in terrestrial N cycling and also is highly sensitive to soil pollutants [174]. The bioreporter strain contains the *luxAB* genes from *V. harveyi* directed by the *hydroxilamine oxidoreductase* promoter (*hao*) from *N. Europaea*, driving a constitutive light emission strongly correlated with respiration [175]. It has been used to assess the toxicity of a linear alkylbenzene sulfonate surfactant (LAS) in soil. The tests were performed in solution and in a solid-phase contact assay, where slurry of the soil and the bacterium was shaken during 1 h and then the luminescence was tested directly or in the supernatant obtained after centrifugation. The toxicity was very similar in solution and in the solid-phase contact, indicating that LAS was highly available. *N. europaea* ATCC 19781 (pHLUX20) was highly sensitive to LAS, whereas heterotrophic soil microorganisms tested by a soil respiration assay were rather insensitive, demonstrating again the importance of testing toxicity with a battery of organisms from different trophic levels. It was also used to test Cu toxicity in soils being useful to support the theory of terrestrial biotic ligand model for metal toxicity, which postulates that toxicity depends on the free metal ion activity in solution and on ions competing for metal sorption to the biotic ligand [176].

P. fluorescens DF57-40E7 was mainly used as a control to correct interferences in luminescence due to turbidity or toxicity in assays involving lights-on *P. fluorescens* DF57-based bioreporter strains harboring the *luxAB* genes fused to a Cu-inducible promoter in monitoring Cu toxicity and bioavailability in soils, also by a solid-phase contact assay [105, 177–179].

3.2 Bioreporters Specially Designed to Assess Bioremediation Programs and Wastewater Toxicity Evaluation

Some of the bioreporters described so far are too sensitive, making them unsuitable for use with highly toxic samples because extensive cell death may happen during the assay. To avoid this, bacteria isolated from sewage or bioremediation processes, which are more resistant to the toxins that are to be tested, have been transformed with *lux* genes to construct lights-off bioreporters.

Burkholderia sp. RASC c2 (previously classified as *Pseudomonas*) is a 2,4-DCP-mineralizing bacterium, which can use it as sole carbon source [180]. It has been chromosomally marked by integration of the Tn4431 transposon, which harbors the *luxCDABE* genes from *V. fischeri* fused to the *tet* promoter, giving a constitutive light emission [180]. Thus, it can be used at the same time to bioremediate a soil or a solution contaminated with DCP and to monitor the bioremediation process [180]. Its toxicity to mono-, di-, and tri-chlorophenols has been assessed, increasing the toxicity with the degree of chlorination; in addition, the toxicity levels in function of toxic concentration correlated better with toxicity to aquatic species, such as *Pimpephales promelas* (fathead minnow) and *Tetrahymena pyriformis* (ciliate), than to *V. fischeri* [181]. Degradation, growth rates, and toxicity to DCP, Zn, and Cu in transformed and untransformed strains were equivalent [182].

Shk1 (Shock 1) lights-off bioreporter was developed by Kelly et al. [183] by transforming an activated sludge isolate bacterium with a plasmid containing the *luxCDABE* genes from *V. fischeri*. Later, this bacterium was identified as *P. fluorescens* [184]. This bacterium is adapted to the activated sludge environment and resists the shock of exposure to this toxic environment better than other bioreporters. This bioreporter responded to DCP, Cd, and hydroquinone in a pH range between 6.1 and 7.9, but not to ethanol concentrations up to 10,000 mg/L [183]; it also responded to a wide array of toxicants in close agreement with respirometry experiments but not with *V. fischeri* tests [185]. It has also been tested with narcotic chemicals [186] and has been used to assess metal mixtures [187]. A method for continuous monitoring of wastewater influent was developed [183]. Later, it was improved to be used routinely in the influent and effluent of wastewater treatment plants with minimal labor and capital investment [188] and with minimal variability between experiments [189, 190]. A bench scale in batch experiments with influent wastewater and activated sludge from a municipal wastewater treatment plant was also developed, proving the responsiveness to heavy metals. In that environment, the bioreporter was more sensitive to Cd and Zn, followed by Cu and then Ni [191]. Later, it was used in combination with another transgenic *lux* expressing *Pseudomonas* spp. isolated from a domestic wastewater treatment plant, denoted as PM6 [185] and with a battery of test species for toxicity testing [192]. There was no general pattern with regards to the relative sensitivities of both strains (Shk1 and PM6) to a group of chemicals, and they performed at least as well as *V. fischeri* for detecting metal toxicity [185].

Acinetobacter sp. DF4/pUTK2 contains the plasmid pUTK2, which harbors the Tn4431 transposon with *luxCDABE* genes from *V. fischeri* fused to a constitutive promoter [193]. The parent strain (*Acinetobacter* sp. DF4) was isolated from industrial wastewater and used previously for phenol biodegradation and detection [194, 195]. In addition, *Acinetobacter* is an ecologically relevant microorganism; it is widespread in nature and can be isolated from water, soil, living organisms, and even human skin. The bioreporter strain has been optimized to detect toxicity to heavy metals in water and wastewater, being more sensitive to Zn, Cd, Fe, Co, Cr, and Cu (in this order) [193]. Later, it was immobilized in Ca-alginate, agarose, and

agar in microtiter plates and the toxicity of phenolic compounds was tested. The best immobilization matrix was Ca-alginate. The bioreporter was stable at 4 °C for 8 weeks, and responded to phenol in tap water up to 400 ppm, while *V. fischeri* displayed negligible luminescence output at values exceeding 150–200 ppm [196].

Stenotrophonomas 3664 and *Alicagenes eutrophus* 2050 are surfactant-resistant bacteria that were also transformed with pUTK2 plasmid to construct the *Stenotrophonomas* 664 (pUTK2) and *A. eutrophus* BR6020 (pUTK2) lights-off strains [197]. These transgenic bioluminescent strains are 400 times more resistant to the nonionic surfactant polyoxyethylene 10 lauryl ether than *V. fischeri* and are useful for toxicity evaluation of remediation processes, which use surfactants for solubilization of hydrophobic pollutants; they have also been used to assess toxicity of nonpolar narcotic compounds and phenol [197].

3.3 Freshwater Bioreporters

Ecologically relevant microorganisms from inland water environments have been transformed to be used as bioreporters in these environments.

Janthinobacterium lividum YH9-RC harbors the *lux AB* genes from *V. harveyi* integrated into the genome through a mini-Tn5 transposon. Its parental strain, *J. Lividum* YH9-R, is a bacterium isolated from a groundwater sample that is rifampicin-resistant. A freeze-drying method in a 384-multiwell plate was developed and a continuous monitoring system using this device and special software, BactoTox®, was patented. The system was 7.8–8.6 times more sensitive to organic compounds and heavy metals than *V. fischeri* in the Microtox™ assay [198].

Cyanobacteria are photosynthetic bacteria ubiquitous in aquatic environments. They are particularly relevant because they are primary producers and are at the base of food webs, being representative of the health of the environment they live in. Two transgenic lights-off bioreporters based on freshwater cyanobacteria that are useful for toxicity testing have been developed. Shao et al. [199] developed a *Synechocystis* sp. strain PCC6803 chromosomally marked with the luciferase gene (*luc*) from the firefly *P. pyralis* fused to a constitutive promoter. This strain was optimized to uptake luciferin, the luciferase substrate, and was sensitive to the herbicides diuron, atrazine, propazine, simazine, paraquat, glyphosate, 4-chloro-2-methylphenoxyacetic acid, and mecoprop, as well as to Cu, Zn, and DCP.

Fernandez-Piñas and coworkers developed an ecologically relevant bioluminescent bioreporter by means of a chromosomal integration of the whole *luxCDABE* operon from *P. luminescens* into *Anabena* sp. PCC7120, a filamentous cyanobacterium. The strain was denoted as *Anabaena* CPB4337 and showed an extraordinary high and stable luminescent signal. Neither growth nor cell viability was affected by the chromosomal integration or the bioluminescence expression [149]. The organism is functional in a wide range of pH conditions: from pH 5 up to pH 9 [60, 200]. It has been demonstrated to have a very good level of inter- and intra-experimental reproducibility [201], and it has been successfully tested in

environmental matrices of different complexity [60, 200, 202, 203]. The organism has been used in combination with a battery of organisms of other tropic levels, such as *V. fischeri*, *D. magna,* and the green alga *Pseudokirchneriella subcapitata* to study the toxicity of different priority and emerging pollutants. The studies showed that *Anabaena* CPB4337 was a very sensitive strain, particularly to emerging pollutants such as fibrates (being for some of them up to two orders of magnitude more sensitive than *V. fischeri*) [204], perfluorinated surfactants and chlorinated by-products [203], antibiotics [205], and nanomaterials [206, 207]. Especially remarkable is the work performed with *Anabaena* CPB4337 in mixture toxicology by this research group, which is reviewed in the Sect. 5.2.

3.4 Eukaryotic Bioreporters

The yeast *Saccharomyces cerevisiae* is a eukaryotic microorganism that is more relevant as a model to test toxicity for human health than bacteria. It is protected by a cell wall that makes it resistant to extreme pH, solvent exposure, and osmotic shock, permits the detection of organic or inorganic toxins in extreme conditions, and also detects compounds that are nontoxic to bacteria but toxic to eukaryotes [208]. Hollis et al. [208] developed a transgenic strain of *S. cerevisiae* W303-1B containing the *luc*Δ (without the peroxisome target sequence) gene from the firefly *P. pyralis* integrated into the genome and expressing it constitutively. *D*-luciferin, the luciferase substrate, has to be added exogenously. It is an amphipathic molecule that has difficulty crossing the cell membrane, so the authors developed a method to acidify the membrane after toxin treatment and before luminescent measurement, adding luciferin with citrate phosphate buffer at pH 2.5. The strain was similarly responsive to the herbicides diuron and mecoprop in a range of pH from 3 to 10, while *E. coli* HB101 (pUCD607) did not respond to these metabolites. It also detected Cu, although not in an extreme pH, probably due to changes in Cu speciation. They also tested the effect of Cu toxicity in different solvents [208].

Later, Gupta and coworkers inserted the *luxA*, *B*, *C*, *D,* and *E* genes from *P. luminescens* in *S. cerevisiae* strains W303a and hER, being the first time that these prokaryotic genes were transformed in a eukaryotic microorganism [209]. Because the genes are not expressed as polycistronic mRNAs in eukaryotic organisms, the genes were transformed independently in two plasmids with bidirectional promoters. One plasmid harbored *luxA* in one direction and *luxB* in the other. The other plasmid carried *luxE* in one direction and *luxC* and *luxD* in the other; *luxC* and *luxD* were separated by the IRES (yeast internal ribosomal entry site) sequence that permits high bi-cistronic expression. Two sets of transformants were made—one with the constitutive promoters from *gliceraldehyde 3-phosphate deshydrogenase* (*GPD*) and *alcohol deshydrogenase* (*ADH1*) genes and the other set with the promoters from *GAL10* and *GAL1* genes, which are inducible by lactose. The strains produced luminescence autonomously, but it was very

unstable, persisting at a maximum level less than 20 s. Then, the authors introduced the *frp* (*flavin oxidoreductase*) gene from V. *harveyi* downstream from the *luxE* gene, both separated by an IRES sequence. This gene codes for a NADPH-dependent FMN oxidoreductase and can regenerate the pool of $FMNH_2$ necessary for prokaryotic bioluminescence. This strain, named BLYEV, yielded stable luminescence at levels similar or greater than prokaryotic bioreporters containing the *luxCDABE* genes. It may have greater or equal potential for environmental monitoring than many prokaryotic bioreporters containing a *luxCDABE* transcriptional fusion [209].

3.5 Aequorin-Based Bioreporters

Intracellular messengers are basic components of signaling systems. Amongst them, calcium has arisen as probably the most versatile one in eukaryotes [210–212] and, as increasing evidence indicates, also in prokaryotes [213]. This versatility is probably derived from the existence of diverse calcium signaling systems with characteristic spatial and temporal properties [11, 210, 211]. In different cell types, a variety of abiotic and biotic stimuli generate intracellular calcium signals. The specificity of the signal relies not only in the change of the intracellular calcium concentration; a combination of changes in all Ca^{2+} parameters of the signal such as amplitude, duration, frequency, rise time, final Ca^{2+} resting levels, recovery time, and source of the signal induced by a specific stimulus is referred to as a Ca signature. The Ca^{2+} signatures encode information relating to the nature and strength of stimuli in their spatial-temporal dynamics [214–218].

The fact that calcium may respond to a variety of environmental stimuli in practically any organism has raised the question of whether calcium might also sense chemicals that might be potentially toxic to the cell. Recently, toxicity bioassays based on the effect of potential pollutants on intracellular calcium dynamics have been developed in different eukaryotic and prokaryotic organisms. The tests are based on another bioluminescent protein, apoaequorin, isolated from the jellyfish *Aequorea victoria* [219]; its gene has been cloned and successfully expressed in animal, plant, and bacterial cells, allowing quantitation of intracellular Ca^{2+} fluxes [213, 216, 218]. Particularly, regarding both prokaryotic and eukaryotic microorganisms, apoaequorin has been expressed in *E. coli* [220], *S. cerevisiae* [221], *B. subtilis* [222], *Phaeodactylum tricornutum* [223], *Anabaena* sp. PCC7120 [216], *Aspergillus nidulans* [224], *Aspergillus awamori* [225], *Dictyostelium discoideum* [226], *Mesorhizobium loti* [227], *Synechococcus elongatus* [228], *R. leguminosarum* [229], *Neurospora crassa* [230], and *Aspergillus niger* [231].

Functional recombinant aequorin can be successfully reconstituted upon addition of the hydrophobic luminophore coelenterazine; the reconstituted protein has three Ca^{2+} binding sites. Once Ca^{2+} ions are bound, aequorin catalyzes the oxidation of the substrate coelenterazine by oxygen, resulting in blue light emission

that can be measured with a luminometer [232]. The amount of luminescence emitted by aequorin is dependent upon free intracellular calcium concentration. Thus, aequorin can be used to monitor intracellular free calcium dynamics in vivo and in a continuous fashion, allowing one to record specific calcium signatures in response to any environmental stimulus, including pollutants. Aequorin is very sensitive to Ca^{2+} changes, with a dose–response curve that begins at around 100 nM free Ca^{2+} and is saturated well above 10 μM free Ca^{2+} [216].

Since the late 1990s, various types of toxicants that may potentially disrupt calcium homeostasis in microorganisms expressing apoaequorin have been tested (Table 2). Many of the tested toxicants are currently considered emerging pollutants or priority pollutants. Normally, these assays relate the toxic substance (antibiotics [86], anti-inflammatory [233], antiarrhythmic drugs [234–236] and antifungals [230, 231, 237]) with modification of processes such as chemotaxis (heavy metals [238]), oxidative stress (H_2O_2 [222, 224] and organic peroxides [239]), and heavy metals [226, 240], which that might influence the signaling of calcium. However, these studies were focused in assessing the relationship between perturbation of intracellular calcium homeostasis and the presence of toxicants; none of them can be considered a toxicity test.

The first toxicity test relating calcium and pollutants is probably that reported by Kozlova et al. [225], which tested the effect of toxic substances (the heavy metals Cr^{6+} and Zn^{2+} and the phenolic polar narcotic 3,5-dichlorophenol) on the dynamics of cytoplasmic free calcium ($[Ca^{2+}]_c$) in the fungus *A. awamori*, transformed with the apoaequorin gene. The authors checked a series of parameters of the calcium signature in the presence of the pollutants: rise time, amplitude, length of transient, final resting level, and recovery time. The toxicants were preincubated 5 and 30 min before the treatment with 5 mM external $CaCl_2$ and measurement of signal. Length of transient (LT_{50}, at the point where amplitude equals half the amplitude maximum) of $[Ca^{2+}]_c$ was chosen to compare with the EC_{50} at 5 and 30 min of the *V. fischeri* classical bioluminescent assay. All toxicants provoked a response in one or more of the studied parameters, but LT_{50} was not the best parameter to compare with the *V. fischeri* bioassay. Therefore, the authors concluded that assessment of a different parameter was needed.

Kozlova patented the method as *eukaryotic biosensor making use of calcium regulated light emitting enzyme* (U.S. Patent Application 20060094002). This method consisted of using transformed cells in which calcium-sensitive photoproteins (such as apoaequorin) have been included for the identification of the pollutants. Proprietary methodology is based on the characteristics of calcium signatures to discriminate between the contaminants, including those in mixtures. Fungi and yeast in addition to other cells that express this protein play a key role detecting contaminants in this patent. Also, in this patent, such aequorin-based assays have advantages over other conventional methodologies. These tests can distinguish between different toxicants in contrast to the conventional systems, which only provide an indication of whether a contaminant is toxic or not. In addition, the aequorin-based assays may provide data on possible mechanisms of action [225].

Table 2 Main features and applications of recombinant bioluminescent microorganisms

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
E. coli HB101 (pUCD607)	Prokaryotic Gram−/ Enterobacteria	*tet-luxCDABE V. fischeri*		Heavy metals and organic compounds in solution, soil extracts, freshwater, and remediation processes. Mixtures. Online continuous biosensing (fingerprinting) and prediction of contaminants in spiked water samples, effluent of a metal plating plant, and a malt distillery	[73, 101, 102, 104, 105, 107–112, 120, 123, 124, 129–132, 135–138]
E. coli MC1061 (pSLlux)	Prokaryotic Gram−/ Enterobacteria	*lac-luxCDABE P. luminescens*		Heavy metals in solution as control of inducible strains and in ecotoxicity. Control of inducible strains to oxidative damage caused by nanoparticles. Zn, Cd, Hg, Cu, 3,5-DCP, aniline, and 3,5-DCA in solution; comparison with *V. fischeri* and *E. coli* (pSLlux)	[146, 147, 152]
E. coli MC1061 (pDNlux)	Prokaryotic Gram−/ Enterobacteria	T7-*luxCDABE P. luminescens*		Control of inducible strain to Hg in soil sediments of mining area immobilized or not in alginate-fiber optic, direct contact with soil. Heavy metals in solution as control of inducible strains and in ecotoxicity. Effects of Rhamnolipids on Cd bioavailability in solution and soil extracts. Zn, Cd, Hg, Cu, 3,5-DCP, aniline, and 3,5-DCA in solution; comparison with *V. fischeri* and *E. coli* (pSLlux)	[146, 147, 151, 153, 175]

(continued)

Table 2 (continued)

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
E. coli GC2	Prokaryotic Gram−/ Enterobacteria	*lac-luxCDABE P. luminescens*		Azo-dyes and γ radiation in solution. Biosensor dispositive immobilized in agar in polypropylene tubes for continuous monitoring of BETEX in air. PAH in soil extracted with rhamnolipids. Portable minibioreactor with freeze-dried cells to detect phenols. Multichannel two-stage mini-bioreactor for continuous monitoring in combination with lights-on strains in rivers and wastewater treatment plant effluents. Immobilized in chip and plate arrays in combination with lights-on strains	[157, 159–166, 169–171]
S. typhimurium TA104 *pr*1	Prokaryotic Gram−/ Enterobacteria	*luxCDABE V. fischeri*	VITOTOX®	Control strain of a lights-on strain in genotoxic assays	[173]
P. fluorescens 10586 (pUCD607)	Prokaryotic Gram−/Soil	*tet-luxCDABE V. fischeri*		Effluent of a malt whisky distillery (Cu) and a papermill treatment plant (Cd and PCP). Arable soils fertilized with paper mill sludge (Cd, Cu, and PCP). Heavy metals and organotins in solution and soils. Herbicides in freshwaters. Remediation assessment of chlorophenols, CAHs, and PAHs	[102, 104, 106, 108, 109, 112, 115–120, 123, 125, 126, 130, 133, 134, 136, 140, 141, 274]
P. fluorescens 10586/ FAC510	Prokaryotic Gram−/Soil	*tet-luxABE V.fischeri*		Heavy metals in soil and solutions, pH, and complexing agents effect	[139–141]
P. fluorescens 8866 Tn5*luxCDABE*	Prokaryotic Gram−/Soil	*luxCDABE V. fischeri*		Cu in soil and solution and Zn and DCP in solution	[142]

(continued)

Table 2 (continued)

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
P. fluorescens DF57-40E7	Prokaryotic Gram−/Soil	*luxAB V. fischeri*		Control strain of one lights-on strain inducible by Cu	[105, 177–179]
P. fluorescens OS8 (pDNlux)	Prokaryotic Gram−/ Toluated contaminated soil	T7-*luxCDABE P. luminescens*		Control of lights-on strains by phenols from oil shale industry in groundwater and semi-coke leachates. Heavy metals in solution as control of lights-on strains and in ecotoxicity. Effects of rhamnolipids on Cd bioavailability in solution and soil extracts	[147, 148, 153]
P. fluorescens OS8::Knlux	Prokaryotic Gram−/ Toluated contaminated soil	*lac-luxCDABE P. luminescens*		Heavy metals in solution as control of inducible strains and in ecotoxicity	[147]
P. fluorescens Shk1	Prokaryotic Gram−/ Activated sludge	*luxCDABE V. fischeri*		Wide array of toxicants, DCP, Cd, hydroquinone, narcotic chemicals, metal mixtures in wastewater influent, and effluent. Batch experiments and continuous monitoring of wastewater	[183–192, 253, 254]
Pseudomonas spp PM6	Prokaryotic Gram−/ Domestic wastewater	*luxCDABE V. fischeri*		Seven metals and 25 organic compounds	[185]
P. putida F1 (pUCD607)	Prokaryotic Gram−/Soil. Toluene degrader	*tet-luxCDABE V. fischeri*		Remediation assessment of oils and PAHs in soil in combination with lights-on bioreporters. Herbicides in freshwaters	[43, 114, 123, 124, 134, 142]
P. putida F1 Tn5l*uxCDABE*	Prokaryotic Gram−/Soil. Toluene degrader	*luxCDABE V. fischeri*		Cu in soil and solution and Zn and DCP in solution. Monitoring the degradation and toxicity of BETEX in soils in combination with a lights-on bioreporter	[142, 143]

(continued)

Table 2 (continued)

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
R. leguminosarum bv *trifolii* (pUCD607)	Prokaryotic Gram−/Soil and plant symbiont	*tet-luxCDABE V. fischeri*		Arable soils fertilized with paper mill sludge (Cd, Cu and PCP). Cd, Ni, Zn, and Cu in solution	[115, 116]
R. *leguminosarum* bv *trifolii* TA1-Tn5l*uxAB*	Prokaryotic Gram−/Soil and plant symbiont	*luxAB V. fischeri*		Heavy metals in soil and solutions; effect of complexing agents. PAHs in solution	[120, 123, 141]
B. subtilis BR151(pBL1/ p602/22lux)	Prokaryotic Gram+/Soil	*lac-luxCDABE P. luminescens*		Heavy metals in solution as control of inducible strains and in ecotoxicity	[147]
B. subtilis BR151(pCSS962/ pBL1)	Prokaryotic Gram+/Soil	*luc*FF *Photinus pyralis*		Control of inducible strains to detect Cd in soils contaminated by metal smelters. Direct contact with soil	[154]
S. aureus RN4220 (p602/22lux)	Prokaryotic Gram+/ Opportunistic pathogen	*lac-luxCDABE P. luminescens*		Heavy metals in solution as control of lights-on strains and in ecotoxicity	[147]
S. aureus RN4220 pTOO02	Prokaryotic Gram+/ Opportunistic pathogen	*luc*FF *P. pyralis*		Control of lights-on strains to detect Cd in soils contaminated by metal smelters. Direct contact with soil	[154]
Streptomyces lividans (pESK004)	Prokaryotic Gram+/Soil	*hao-luxAB V. fischeri*		Heavy metals, chlorophenols, and pesticides in solution and natural samples	[156]
N. europaea ATCC 19781 (pHLUX20)	Prokaryotic gram−/Soil nitrificant	l*uxAB V. harveyi*		LAS and Cu in soil in solid-phase contact. Ammonia monooxigenase inhibitors	[174–176]

(continued)

Table 2 (continued)

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
Burkholderia sp. RASC c2	Prokaryotic /Soil Gram− DCP mineralizing bacterium	*tet-luxCDABE V. fischeri* integrated in genome		Mono-, di-, and trichlorophenols in bioremediation processes. Zn and Cu	[180–182]
Acinetobacter sp. DF4/pUTK2	Prokaryotic Gram− / Widespread. Industrial wastewater isolate	*luxCDABE V. fischeri* in a plasmid		Phenol and heavy metals in water and wastewater. Batch cultures and immobilized in Ca-Alginate	[193, 196]
Stenotrophomonas 664 (*pUTK2*)	Prokaryotic Gram−/ Industrial landfill leachate	*luxCDABE V. fischeri*		Polar narcotic compounds and phenol in remediation processes with surfactants to solubilize pollutants	[197]
A. eutrophus BR6020 (PUTK2)	Prokaryotic Gram−/ Activated sludge	*luxCDABE V. fischeri*		Polar narcotic compounds and phenol in remediation processes with surfactants to solubilize pollutants	[197]
J. lividum YH09-RC	Prokaryotic Gram−/ Groundwater isolate	*luxAB V. harveyi*	Bactotox®	Phenol, benzene, toluene, and heavy metals in solution. Wastewater samples. Freeze-dried immobilized in 384-multiwell plate for a continuous monitoring system	[198]
Anabaena sp. PCC7120 CPB 4337	Prokaryotic Gram−/ Photosynthetic freshwaters	*luxCDABE P. luminescens* integrated in genome		Priority and emerging pollutants, perfluorinated surfactants, chlorinated by-products, antibiotics, and nanomaterials in aqueous solution and in wastewater effluents	[60, 200–207, 344]

(continued)

Table 2 (continued)

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
Synechocystis sp PCC6803	Prokaryotic Gram−/ Photosynthetic freshwaters	*tac-luc P. pyralis*		Herbicides, Cu, Zn, and DCP in aqueous solution	[199]
S.cerivisiae W303-1B LucΔ	Eukaryotic (yeast)	LucΔ *P. pyralis*		Herbicides diuron and mecoprop in solution. Cu in aqueous solution and different solvents	[208]
S. cerivisiae BLYEV	Eukaryotic (yeast)	*Lux A, B, C, D, E* from *P. luminescens* and *frp* from *V. harveyi*		Although not used in environmental monitoring yet, this strain has greater or equal potential than many prokaryotic bioreporters containing a *luxCDABE* transcriptional fusion	[209]
E. coli AB1 157 (pMMB66EH-AEQ)	Prokaryotic Gram−	Aequorin		Heavy metals (Disruption of Ca^{2+} homeostasis)	[238]
Anabaena sp. PCC 7120 (pBG2001a)	Prokaryotic Gram−/ Freshwater	Aequorin		Heavy metals, pharmaceuticals, solvents, naphtalene binary mixtures, complex mixture and real wastewater sample (Recording of specific Ca^{2+}signatures upon exposure to pollutants)	[355]
S. cerevisiae H208-3B (pGAPAQ1)	Eukaryotic (yeast)	Aequorin		Salicylic acid	[233]
S. cerevisiae JK9-3da (pEVP11/ AEQ)	Eukaryotic (yeast)	Aequorin		Eugenol (antifungal)	[237]
S. cerevisiae BY4742 (pEVP11-Aeq89)	Eukaryotic (yeast)	Aequorin		Amiodarone (antiarrhythmic drug)	[234–236]

(continued)

Table 2 (continued)

Organism	Organism type/ Origin	Gene system	Commercial tests	Applications	References
S. cerevisiae H208-3B (pGAPAQ)	Eukaryotic (yeast)	Aequorin		Azalomycin F (antibiotic)	[86]
S. cerevisiae BY4742 (pEVP11-Apoaequorin)	Eukaryotic (yeast)	Aequorin		As (Disruption of Ca^{2+} homeostasis)	[240]
N. crassa	Eukaryotic (Fungi)/Soil	Aequorin		PAF (antifungal) (Disruption of Ca^{2+} homeostasis)	[230]
A. niger	Eukaryotic (Fungi)/Soil	Aequorin		AFP (antifungal) (Disruption of Ca^{2+} homeostasis)	[231]
A. awamori 66A	Eukaryotic (Fungi)/Soil	Aequorin		Cr, Zn (heavy metal) and -3-5-DCP (Recording of specific Ca^{2+} signatures upon exposure to pollutants)	[225]

In 2010, aequorin was used as a reporter in *E. coli* to detect toluene and similar compounds [241]. The biosensor was constructed based on a transcriptional fusion of the lower pathway promoter (Pu) of the *xyl* operon of *Pseudomonas putida* mt-2 aequorin cDNA to apoaequorin cDNA; transcriptional activator xylR was also incorporated. Binding of xylR protein to a subset of toluene-like compounds activates transcription at the Pu promoter; thus, expression of aequorin is controlled by xylR and Pu. Toluene, benzene, and xylene induced the biosensor response. The detection range for toluene was from 1 to 500 μM. The bioluminescence response was specific for toluene-like molecules, so this biosensor cell would be able to accurately detect toluene derivative contamination in environmental samples.

Barran-Berdon et al. [242] proposed that intracellular free Ca^{2+} might serve as an early biomarker of exposure to environmental pollution by using the cyanobacterial bioreporter *Anabaena* sp. PCC 7120 (pBG2001a) constitutively expressing apoaequorin [216]. The authors recorded and analyzed the Ca^{2+} signatures generated by exposure of the cyanobacterium to different groups of environmental pollutants, including metals, organic solvents, naphthalene, and pharmaceuticals. They found that, in general, each group of tested chemicals triggered a specific calcium signature in a reproducible and dose-dependent manner. The authors also recorded Ca^{2+} signals triggered by binary mixtures of pollutants and a signal induced by a real wastewater sample, which could be mimicked by mixing its main constituents. Finally, they hypothesized that these Ca^{2+} signals might be related to the cellular mechanisms of pollutant perception and ultimately to their toxic mode of action.

Bioassays using microorganisms expressing apoaequorin will be essential in future ecotoxicology because they provide deeper and more complex information than classical toxicology tests. Table 2 provides a summary of recombinant luminescent microorganisms, featuring the species, gene system, type of organism, commercial devices if available, and main applications.

4 Relevance of QSAR Models and Hormesis in Ecotoxicology

4.1 Bioluminescent Microorganisms and QSAR Models

Quantitative structure–activity relationship models (QSAR models) are regression models used in natural sciences (e.g., chemistry, biology or pharmacology) in order to relate a set of *predictor variables* (x) to the potency of the *response variable* (y). The predictor variables are linked to physico-chemical characteristics of the chemical compounds, such as K_{ow} (octanol/water partition coefficient), molar volume, molar refractivity, and substituting chemical features. The response variable can be a biological parameter, such as citotoxicity expressed as bioluminescence inhibition. QSAR modeling tries to find a relationship between chemical descriptors

and the biological endpoint studied, allowing prediction of the biological activity of new chemicals based on the information of their theoretical descriptors. Its final goal is to generate toxicological data in silico for new chemicals and anticipate their toxicity based on their physico-chemical descriptors [243, 244].

Bioluminescent microorganisms have been extensively used in QSAR modeling [244–247]. Based on their ease of use and high-throughput configuration, they have been used to construct databases of biological responses to chemicals. These biological data, combined with measured or computed *chemical predictors* of the molecules, allowed several applications that have been very useful in ecotoxicology. QSAR modeling has allowed finding correlations between toxicity and basic physicochemical parameters of organic chemicals and their mechanism of toxic action (MOA), such as K_{ow} or the highest occupied molecular orbital and lowest unoccupied molecular orbital (HOMO and LUMO, respectively) [244]. Molecules whose toxicity show a high correlation with K_{ow} have been assigned to the *non-polar narcosis* mode of action (an unspecific mode of action related with the accumulation of the chemical in the biological membranes causing their disruption). In this line, Hermes et al. [246] found that K_{ow} together with other chemical descriptors was able to explain the toxicities of 22 organic chemical to *V. fischeri*. Croning et al. [248] found that K_{ow} explained the toxicity of alkanones to *V. fischeri*, and Yu et al. [249] found that the toxicity of halogenated benzenes and their mixtures to *V. fischeri* could be assigned to the nonpolar narcotic mechanisms of action.

The chemical descriptors *energies of HOMO and LUMO* are related to the chemical reactivity of the molecules. They have been found to explain properly the toxicity of organic chemicals with mechanisms of action in which chemical reactivity is important, such as weak acid respiratory uncouplers (WARUs) and electrophile/proelectrophile reactants. Croning et al. [248, 250] identified aldehydes exerting their toxicity to *V. fischeri* as *Schiff based-forming electrophiles* and alkenals as *michaelis-type acceptor electrophiles*. Yuan et al. [251] found that 2,4 dinitrotoluene and several aromatic compounds (nitrobenzenes and anilines) were also well explained by the *LUMO* chemical descriptor of the molecules and identified their toxicity acting by an intracellular oxidation MOA. However, since the year 2000, chemical descriptor-based QSAR models started to show some weaknesses, such as their inability to predict with an unique QSAR model the toxicity of chemicals acting by different modes of action (as polar and nonpolar narcotics or electrophilic reactants), and the requirement of measuring physicochemical properties of the compounds previous to the QSAR modeling. This is a real disadvantage when studying a very large number of family-related chemicals; for example, there are more than 1 million ionic liquids [252].

The efforts of researchers have focused on the development and testing of new QSAR strategies and variable selection methods based on new mathematical methodologies. Ren et al. [253, 254] proposed the introduction of a solvation parameter in order to be able to predict the toxicity of polar and nonpolar narcotics with the same QSAR model. They were able to model the toxicity of 98 organic chemicals to the recombinant bioluminescent bacteria *Pseudomonas* Shk1 and *V. fischeri*. Between new QSAR approaches, *fragment-based* QSARs gained

popularity in ecotoxicology due to their high predictive potency [244, 245, 252, 255–258]. Fragment-based QSARs work on the premise that a molecule can be described by the sum of its fragments. This basic concept can be applied by analogy to generate in silico chemical descriptors. For example, the partition coefficient of a compound can be estimated as a function of the partition coefficient of its fragments instead of measuring it experimentally.

In 2002, Khadikar et al. [257] developed a QSAR model based on a topological index (named *Id*) to model both the hydrophobicity and the toxicity to *D. magna*, *F. minnow,* and *V. fischeri* of *halo-* and *methyl-* substituted benzene derivatives. They found that even monoparametric QSAR models based on *Id* gave excellent results, modeling the hydrophobicity and the toxicity of such chemicals. Agrawal et al. [255] used molecular connectivity indices based on narcotic mechanism of action to explain the toxicity of 39 organic chemicals to *V. fischeri;* they showed that the topological index together with indicator variables accounting for information on the degree of branching and substructural features gave good estimates of the measured toxicity.

Klopman et al. [244, 259, 260] published a series of monographs on the application of one of the first commercially available toxicological QSAR modeling PC programs for users (M-CASE, http://www.multicase.com/products/products.htm). The program operates on the basis of fragment-based QSAR to find chemical substructures that correlate (positively or negatively) with biological activity, including some *modulators* to refine the prediction (which can include additional active structural fragments or physical properties such as K_{ow} or *HOMO/LUMO* chemical descriptors). They studied several utilities of M-CASE with data sets of several relevant organisms. In aquatic toxicity, they studied its applicability with a data set of the toxicity of 901 chemicals to *V. fischeri* [244]. M-CASE was able to predict the toxicity of the tested chemicals and it was able to identify the main *toxicophores* (chemical fragments that increased toxicity) and *biophobes* (chemical fragments with negative correlation with toxicity) and to assign them their principal mode of action (polar and nonpolar narcosis, ester narcosis, WARUs, and electrophiles/proelectrophiles). Also, since 2003, there has been an increasing interest in joining both in silico chemical descriptors and toxicity of mixtures of chemicals [244, 261, 262]; this topic will be reviewed in the Sect. 5.2 of this chapter.

With the increasing number of available descriptors, the development of variable selection methods dealing with the selection of the best subset of chemical descriptors/parameters to explain the biological response started to gain interest. Roy et al. [258] applied a *genetic function approximation* with an extended topochemical atom index to model the toxicity of phenylsulfonyl carboxylates to *V. fischeri*. Melagraki et al. [263] developed a novel neural network training methodology based on a radial basis function to predict the toxicity of 39 heterogeneous sets of chemicals to *V. fischeri*. The model worked faster and was significantly more accurate than traditional QSAR models. Asadollahi-Baboli [264] explored the applicability of machine learning methods in QSAR for the analysis of the toxicity of phenols and thiophenols to *V. fischeri*.

With the increasing potency of QSAR methods, researchers were able to study more chemicals in greater detail. Luis et al. [252] focused on the development of a new method to predict the contribution of different functional groups to the toxicity of ionic liquids to *V. fischeri*. They studied a thousand parental ionic liquids. The method allowed estimation of the contribution of different combination of functional groups to the overall toxicity of the compounds. Some conclusions of the studies were that, in general, cationic functional groups increased the toxicity (pyrrolidinium, imidazolium, and pyridinium groups contributed about 3, 20, and 33 % to the toxicity, respectively) and anionic groups reduced toxicity—except for the dycianamide and ethylsulfate groups, which were found to be very toxic to *V. fischeri*. Aruoja et al. [245] studied the toxicity of 58 substituted anilines and phenols to *V. fischeri* and other organisms. They found that the occupied *para*-position tended, in general, to increase toxicity, whereas *ortho*- substituted congeners showed a reduced toxicity.

However extensive the applications in the study of the toxicity of new chemicals and the influence in toxicology of the variations in chemical motives, more basic and field applications of QSAR modeling were not common until very recent years. Kim et al. [265] studied the ecological risk of acetaminophen, carbamazepine, diltiazem, and six major sulfonamides using *D. magna, Orycias lapiles and V. fischeri*. They found that the toxicity was explained by the physicochemical descriptors related with chemical reactivity (*EHOMO/ELUMO*). Vighi et al. [247] compared the dependence on narcosis and polar narcosis of the toxicity induced by regulated chemicals on *V. fischeri* to that of other model organisms (algae, Daphnia, and fish). The study concluded that bacteria (represented by *V. fischeri*) responded similarly to eukaryotes (algae, Daphnia and fish) and nonpolar narcotics; however, *V. fischeri* was two orders of magnitude more sensitive than Daphnia and fish to polar narcotics.

Escher et al. [266] studied the influence of membrane-water partitioning, membrane permeability, and baseline toxicity of the parasiticides ivermectin, albendazole and morantel, to *V. fischeri* and green algae. Qin et al. [267] studied the comparative toxicity of several environmentally relevant organic pollutants to seven aquatic organisms (including *V. fischeri*). Jiang et al. [268] and Zou et al. [269] predicted the toxicity of antibiotics on *P. phosphoreum* and modeled the chronic toxicity based on acute toxicity data. Chen et al. [270] generated a QSAR model to predict the toxicity of 95 dioxins and dioxin-like compounds to *V. fischeri.*

Despite their high predictive capacity and the possibility to generate purely in silico predictions—not only of the toxicity of chemical compounds, but also of the chemical descriptors explaining the toxicity—the application of QSAR models is still stifled by many problems inherent in developing and validating QSAR models for toxicology. These problems include the use of inappropriate molecular descriptors and nontransparent computational tools, the undetected existence of chemicals and chemical features that cause large changes in toxicity with only small differences in molecular structure ("acting cliffs"), spurious correlations, lack of quality control in toxicity data, and overreliance on complex mathematics and statistics [243].

4.2 Hormetic Response of Bioluminescent Organisms and Its Implications in Ecotoxicity Testing

Hormesis can be defined as a biphasic dose–response phenomenon characterized by low-dose stimulation and high-dose inhibition of the studied toxicity endpoint by a given toxicant. It can be also regarded in a temporal context as a time-dependent dose–effect response with a temporal evolution from stimulation to inhibition [271]. Hormesis has been reported in literature of almost every discipline involved in studying living organisms, from human to bacteria and from medicine to plant physiology, including toxicology [271, 272]. However, despite its high reported occurrence, it is a phenomenon that is still poorly understood regarding both its biological meaning, its degree of actual generalization, and its predictability of occurrence [273]. There is an open discussion on the benefits and problems of its inclusion in practical disciplines concerning health and the environment, such as in risk-assessment procedures and models, because the underlying biological meaning of the stimulatory response (beneficial effects/pretoxicological signals) is still unclear. It can be regarded both as a beneficial effect that may be actually investigated or as an early indicator of an actual toxicity phenomenon [271, 274, 275]. However, in light of the latest trends in ecotoxicology, where researchers are trying to gain insight into what is happening at very low concentration of contaminants (low to no-effect levels) and the increasing attention to perform risk assessment at environmental concentrations of pollutants (concentrations where hormesis is rather possible to occur), we have included a brief discussion of what is known about hormesis in bioluminescent organisms.

Stimulation of the bioluminescence response depending on external factors appears as an intrinsic phenomenon of bioluminescence, and it is present in many published studies involving naturally bioluminescent organisms [60, 203, 276, 277]. It is difficult to clearly differentiate hormesis from simple stimulation of bioluminescence induced by a series of environmental factors such as temperature, pH, and nutrient ions (e.g., Na^+, Ca^{2+}, Cl^-, PO_4^{3-} or CO_3^{2-}) [60, 278, 279]. In fact, hormesis has been scarcely studied in a systematic way, neither identifying the chemical or physical agents that lead to the hormetic responses and its reproducibility, nor determining the biological meaning of hormesis in bioluminescent organisms. To our knowledge, there are no reviews of hormesis in bioluminescent organisms, and few works are available focusing on the hormetic response. However, some examples can be found.

Chistofi et al. [280] studied the hormetic response of *V. fischeri* exposed to heavy metals (Cr, Zn, Cu) and some organics (2,4,5-trichlorophenol and 3,5-dichlorophenol and phenol). They found a clear time-dependent hormetic response of the bioluminescence of *V. fischeri* when exposed to sublethal concentrations of Zn, Cr, Cd, and 3,5-DCP. They even described an undulant response to Cu as a function of time, which they identified as a γ-type hormetic dose–response curve [280]. They also reported in a related study a hormetic response of *V. fischeri* when exposed to

waste water samples [281]. Ward et al. [277] reported similar results using *V. fischeri* to study the toxicity of leachates from Florida municipal soil waste landfills.

Fulladosa et al. [276, 282] studied the apparent resistance of *V. fischeri* to inorganic and organic forms of As. They found a clear hormetic response of the bioluminescence when the organism was exposed to As and arsenobetaine and monomethylarsonic acid. These studies allowed them to find a resistance mechanisms to As in *V. fischeri* responsible for its high tolerance to arsenical compounds [283]. Czyz et al. [284] found an increase in bioluminescene of *V. fischeri* when irradiated with ultraviolet light and some chemical mutagens, such as sodium azide (SA), 2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine x 2HCl (ICR-191), 4-nitro-*O*-phenylenediamine (NPD), 4-nitroquinolone-*N*-oxide (NQNO), 2-aminofluorene (2-AF), and benzo[alpha]pyrene. Rodea-Palomares et al. found a stimulation of the bioluminescence signal in the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337 exposed to sublethal concentrations of heavy metals [60], lipid regulators [203], perfluorinated surfactants, chlorinated pollutants [206], and cerium oxide nanoparticles [206].

Despite its evident occurrence and its implications for risk assessment at low exposure levels, hormesis continued unconsidered in most of the current risk assessment methodologies, in part due to some difficulties dealing with low effect level. A hormetic result may be dismissed as experimental error or a distortion of results; commonly, data showing a stimulation in the response to toxicants are usually ignored in the toxicity calculations. Experimental design can also distort the observation of hormesis when the range of concentrations of toxicants is too wide, missing the hormetic concentration region [280, 281]. Finally, one of the most evident limitations when dealing with hormesis is the inadequacy of the available mathematical tools to study hormetic dose-response curves and to integrate them in the current models to study toxicity.

However, clear advances have been made in the last decade on the mathematical tools available to study hormesis. From the early works of Van Ewijki and Hoekstra [285] and Brain and Courses [286], the recent contribution to the study of hormesis in bioluminescent organisms made by several researchers [287, 288] has provided the necessary mathematical tools to fit hormetic dose-response curves and statistical testing for significance. Also especially remarkable are the recent contributions made by researchers from Tonji University (Shanghai) to the study of hormetic response in bioluminescent organisms. They have systematically studied *j*-shaped hormetic dose-response curves in *P. phosphoreum* and *V. quighaiensis sp.*-Q67 [64, 289, 290]. They identified a reproducible hormetic effect of some chemicals, such as ionic liquids on the studied organisms. They have improved the available tools to study hormesis to properly fit dose-response models to the *j*-shaped curves by a seven-parameter Weibull equation so that many different types of hormetic dose-response curves can be properly fit. They also worked on the combined effect of substances that depict hormetic dose-response curves when applied individually and were able to estimate the expected responses when they were applied in combination [290].

Concerning the physiological/biochemical causes of the hormetic response in bioluminescent organisms, some authors tried to link hormetic/stimulating response of bioluminescence with a direct effect of the external factor on the luciferase enzyme or the *lux* genes regulation system [284, 289]. In our opinion, there is another possible explanation linked with the general theory of hormesis; the induction in bioluminescence might be considered as a direct reflection of the hormetic/stimulating effect of the external factor on the metabolism of the organism. This explanation matches better with the finding of bioluminescence stimulation occurring both in natural and recombinant bioluminescent organisms with rather different substances (heavy metals and organic chemicals) and even waste waters. However, the discussion is ongoing and more data are needed to clarify the biological meaning of hormesis.

5 Use of Bioluminescent Microorganisms to Assess Mixture Toxicity: From Whole Toxicity Determination to Development of Conceptual Models to Study the Toxicity of Complex Mixtures of Pollutants

Pollutants in the environment do not appear singly but in combinations or mixtures of highly variable degrees of complexity. Furthermore, due to their residual nature, pollutants of different origins can mix and can be biologically or physically/chemically degraded or altered, resulting in one of the most highly complex systems to be studied. Luminescent microorganisms have been extensively used in assessing the toxicity of these complex systems. They are especially well suited for that purpose for three main reasons: their high degree of unspecific response (they can respond to any analyte or physical change in their environment, which may drive a stress perception by the organism), their ease of use, and their fast response [291]. The study of the toxicity of complex mixtures follows two inverse experimental approaches to the complexity issue, which, however, complement each other:

(1) From complex mixture effects to individual components effects, is it possible to identify the chemicals responsible of the toxicity found in a complex mixture?
(2) From individual effect to complex effects, is it possible to predict the toxicity of a complex mixture based on the knowledge of the information of the toxicity of individual components?

5.1 Whole Toxicity Assessment

One of the main advantages of biotoxicity tests (and among them, those using bioluminescent microorganisms) is their ability to respond to any toxic substance present in a sample. Its origin, chemical composition, or other features do not

matter; the only important issue is its toxicity. Based on this advantage, bioluminescent microorganisms quickly found applications in the assessment of the toxicity of complex environmental samples. Examples of the direct assessment of the toxicity of environmental samples with bioluminescent organisms can be found in former works where they were used to study the toxicity of industrial effluents, contaminated soils, or sediments [292, 293]. From these first studies, there has been an increasing body of literature concerning whole toxicity assessment [294–300]. However, gaining knowledge of the toxicity of a sample did not reveal any information about the *actors* of that toxicity.

With the proper development of ecotoxicology, analytical chemistry, and mixture toxicology, researchers began to be more ambitious in their experimental objectives, and the direct assessment of toxicity of environmental samples diversified in related applications. Bioluminescent microorganisms became popular in the monitoring of the efficiency of decontamination processes because they can effectively monitor whether or not the bioremediation process is effectively reducing the toxicity of the remediated element (soil, water, sediment, etc.) [297, 301–303]. In such applications, bioluminescent test systems made evident their advantage as a clear complement to physicochemical determinations. They were able to detect the toxicity not only of the chemical targeted by the remediation process, but of any other unknown substance that may be present or appear during the decontamination. In many cases, an apparent increase in toxicity was observed even when the targeted contaminant/contaminants were effectively removed [73, 297], allowing the identification of a highly relevant phenomenon for environmental toxicology: the occurrence of secondary metabolites and reaction intermediates undetected by instrumental techniques but actually detected as toxic by reporter organisms.

Related to this application, a huge effort has been made in order to establish causal relationships between chemicals that are supposed to be responsible for the toxicity found in vitro and the occurrence and toxicity of those chemicals in the environment (either in wastewater effluents or in other environmental samples such as water, sediments, etc.). This discipline is known as risk assessment; it tries to estimate the environmental risk posed by a substance by taking into account not only its toxicity, but also its occurrence and exposure pathways in the natural environment. In risk assessment studies, a potent analytical capacity is required. Good examples of this kind of analyses are the works by Castillo and Barceló [304], Farré et al. [1, 305, 306], and Ocampo-Duque et al. [299], which targeted the contribution of different particular contaminants (priority pollutants, persistent organic pollutants, and emerging pollutants) to the risk assessment of multipolluted river basins based on a potent analytical capacity, well-designed sampling programs, and the ecotoxicological characterization of the environmental samples using both in situ (biodiversity indexes) and ex-situ toxicity assays (including bioluminescent microorganisms).

Other studies, using a similar operational methodology, have focused on the identification of the actual origin of the toxicological properties found in environmental samples. In this context, special attention is deserved for the so-called

effect-based identification of key toxicants. This method was developed by Brack and coworkers [5, 307, 308] in the context of an EU Research project that started in 2005 within the 6th Water Framework Directive, named Models for Assessing and Forecasting the Impact of Environmental Key Pollutants on Freshwaters and Marine Ecosystems and Biodiversity (MODELKEY) (http://www.modelkey.org). MODELKEY "comprises a multidisciplinary approach aiming at developing interlinked tools for an enhanced understanding of cause-effect-relationships between insufficient ecological status and environmental pollution as causative factor and for the assessment and forecasting of the risk of key pollutants on fresh water and marine ecosystems" [307].

Effect-based identification of key toxicants consists of the identification of key toxicants by a sequential procedure where both analytical chemistry and biological models play a crucial role. First, environmental samples are checked for toxicity and specific unwanted activities, such as endocrine disrupting capacity, by using an in vitro test (including bioluminescent organisms). Once toxicity or dangerous activity is detected, a series of fractional separation of the chemicals present in the sample is performed. Fractions are faced against to the biological panel organisms in order to know the quantitative contribution of each fraction to the total toxicity found. The fractions that continue exerting toxicity are submitted again to fractionation until the chemical responsible for the toxicity is identified [307].

Effect-based identification of key toxicants, together with the other tools developed by the MODELKEY project, have allowed the authors to identify new unknown active chemicals responsible of the toxicity found in natural systems, such as substituted phenols, natural or synthetic estrogens and androgens, dinaphthofurans, 2-(2-naphthalenyl)benzothiophene, and *N*-phenyl-2-naphthylamine [308], as well as to propose a new prioritization of chemicals in the EU based on their ecotoxicological risk [309].

5.2 *Bioluminescent Microorganisms and Toxicology of Mixtures*

One of the key aspirations of mixture (eco)toxicology has been to anticipate quantitatively the effects of both single compounds and mixtures of chemicals. But how is this done? QSAR modeling and toxicology of mixtures are the two tools available for this purpose. Toxicology of mixtures intends to predict the combined toxicity of a mixture of chemicals based on the information of the individual toxicity of its components. Its ultimate goal is to be able to predict the combined toxicity of any environmental mixture based on the information and conclusions obtained by studying a limited number of mixtures [310]. As in the case of QSAR models, the development and testing of models to study the combined toxicity of mixtures has been assisted by the use of bioluminescent organisms.

From ancient times, pharmacology has realized the existence of beneficial and detrimental interactions between drugs, called synergisms and antagonisms [311, 312]. With the expansion of medicine, toxicology, and related sciences, the universality of the occurrence of interactions when an organism is exposed to a mixture of chemicals or active compounds has become a reality [311, 312]. When analyzing the potential biological responses to a combination of chemicals, there exist three possibilities:

(1) Additivity or no interaction, when the biological response to the combination of the chemicals is that expected based on the sum of their individual effects
(2) Synergism, when the biological response to the combination of the chemicals is higher than that expected based on the sum of their individual effects
(3) Antagonism, when the combination of the chemicals is lower than that expected based on the sum of their individual effects.

Synergism and antagonism may be considered as departures from additivity, but what is the definition of additivity when considering the combined effect of two or more chemicals? There has been a lot of controversy on this issue from the first works on the combined effect of drugs from Bliss [313] and Loewe [314] due to the misleading nature of the concept, but it is now clear that additivity is not the simple arithmetic sum of the individual responses [312]. Here, the key element is the definition of the expected response under additivity. Ecotoxicology has made a very valuable contribution to the development and maturation of the general theory of mixtures because it is much easier to test hypotheses in biological systems with faster responses than those used in medicine and human toxicology. Here again, bioluminescent microorganisms have helped in that development [310]. Prior to the review of the contribution to mixture toxicology performed by researchers working with bioluminescent microorganisms (the main body of the present section), we are going to briefly describe and summarize the commonly accepted definitions of additivity.

Basically, two definitions of additivity exist: *Bliss additivity* [313] and *Loewe additivity* [314], which evolved to the so-called concentration addition (CA) (or dose–addition model) and independent action (IA) (or response addition/response multiplication) [315]. The CA model assumes that the chemicals have the same MOA. The joint effect of such kind of chemicals is therefore expected to be equal to the effect of the sum of the chemicals, taking into account the shape of the dose-effect curves of the individual chemicals. The general equation of the CA model is shown in Table 3. On the contrary, IA assumes that chemicals under consideration have different MOAs. The toxic response of each chemical is thought of as a biologically and statistically independent event [316]. IA is based on the multiplication of the expected effects of individual chemicals according to the equation for the generalized IA model, shown in Table 3. These two basic models have been used, adapted, reviewed, and discussed during the twentieth century and discussion continues in the twenty-first century, both from theoretical and practical viewpoints [311, 312, 315, 317–320]. Table 3 summarizes the principal features and equations of CA and IA models, together with those of other models that have

Table 3 Commonly used models to analyze mixture toxicity in ecotoxicology. Models are presented with their additivity definition, main equations, and applicable principal assumptions

Model	Additivity definition	Two-component equation	Generalized equations	Direct statistical testing	Model assumptions	Ref.
CA[a]	Loewe	$\frac{c_1}{\mathrm{EC}x_1} + \frac{c_2}{\mathrm{EC}x_2} = 1$	$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}x_i} = 1$; $\mathrm{EC}x_{\mathrm{mix}} = \left(\sum_i^n \frac{p_i}{\mathrm{EC}_{xi}}\right)^{-1}$	No	Knowledge of individual component dose-response curves is required Components must have the same MOA	[319]
CI[b]	Loewe	$\frac{D_1}{Dx_1} + \frac{D_2}{Dx_2} = \mathrm{CI}$	$\sum_{i=1}^{n} \frac{D_i}{Dx_i} = \mathrm{CI}_X$; $\mathrm{EC}x_{\mathrm{mix}} = \left(\sum_i^n \frac{p_i}{\mathrm{EC}_{xi} \times \mathrm{CI}_{x\mathrm{comp}}}\right)^{-1}$;	No	Knowledge of individual component dose-response curves is required No assumption is made on MOA	[205, 312]
Gennings[c]	Loewe	$g(\mu) = \beta_0 + \beta_1 x_1 + \beta_2 x_2$	$g(\mu) = \beta_0 + \sum_{i=1}^{n} \beta_i x_i$	Yes	Knowledge of individual component dose-response curves is required No assumption on MOA	[321]
TUs[d]	Loewe	$\mathrm{TU}_{\mathrm{mix}} = \mathrm{TU}_1 + \mathrm{TU}_2$	$\mathrm{TU}_{\mathrm{mix}} = \sum_{i=1}^{n} \mathrm{TU}_i$	No	Knowledge of individual component dose-response curves is required Components must have the same MOA	[320]
TEQs[e]	Loewe	$\mathrm{TEQ}_{\mathrm{mix}} = \mathrm{TEQ}_1 + \mathrm{TEQ}$	$TEQ_{\mathrm{mix}} = \sum_{i=1}^{n} TEQ_i$	No	Knowledge of the EC_{50} of individual components is required Identical shapes of dose-response curves of individual components are assumed	[310]
IA[f]	Bliss	$E(c_{\mathrm{mix}}) = E(c_1) + E(c_2) - E(c_1)E(c_2)$	$E(c_{\mathrm{mix}}) = 1 - \prod_{i=1}^{n} (1 - E(c_i))$	No	Knowledge of individual component dose-response curves is required Components must have totally dissimilar MOAs	[319]
INFCIM[g]	Loewe and Bliss	Not applicable	$\mathrm{EC}_{x,\mathrm{mix}} = \frac{1}{\alpha_{\mathrm{sim}}} \left[\frac{1}{\sum_{i=1}^{n} \left(\frac{p_i}{EC_{xi}}\right)}\right] + \alpha_{dis}\left[1 - \prod_{i=1}^{n} (1 - E(c_i))\right]$	No	Knowledge of individual component dose-response curves is required Knowledge of the components' MOA is required	[335]

(continued)

Table 3 (continued)

Model	Additivity definition	Two-component equation	Generalized equations	Direct statistical testing	Model assumptions	Ref.
MIM[h]	Additivity is not considered	$E = f[c, (a_1 * p_i + b_1), ((a_2 * p_i + b_2))]$	Not reported	No	Knowledge of individual dose-response curves is not required Dose-response curves are required for mixtures with at least three different ratios of the same components It is applicable only for two component mixtures; however, a complex mixture can be analyzes as an individual component	[66]

[a] Where c_1 and c_2 are the concentrations of the components 1 and 2 in the mixture producing a fractional effects x (x %). EC_{x1} EC_{x2} are the concentrations of the components 1 and 2 that produce a fractional effect x (x %) individually. In the general equation, c_i is the concentration of ith component in the mixture, EC_{xi} in the concentration of the ith component that produces a fractional effects x (x %) individually. The second equation is the same equation rewritten in the predictive form Faust et al. [319]. Where $EC_{x\text{mix}}$ is the concentration of the mixture producing a fractional effects x. c_i can be rewritten as $p_i^* c_{\text{mix}}$ where p_i is the molar ratio of the ith component and $c_{\text{mix}} = \sum c_i$

[b] Where D_1 and D_2 are the doses of the components 1 and 2 in the mixture producing a fractional effects x (x %). $(D_x)_1$ and $(D_x)_2$ are the doses of the components 1 and 2 that produce a fractional effect x (x %) individually. *CI* is the Combination Index, where $CI < 1$ indicates synergism, $CI = 1$ indicates additivity, and $CI > 1$ indicates antagonism. In the general equation, D_i is the dose of the ith component in the mixture producing a fractional effects x (x %). $(D_x)_i$ is the dose of the ith components that produce a fractional effect x (x %) individually. As can be seen, *CI* model is equivalent to CA model, in fact CA model is a simplification of the CI model where CI is fixed as CI = 1 (additivity). CI equation can also be rewritten in a predictive form Gonzalez-Pleiter et al. [205]. Terms in the equation are the same that in footnote a

[c] Where μ = is the mean response and g(μ) is a specified transformation of the mean known as the link function, x_i = is the dose of chemical i, β_0 is an unknown parameter associated with the intercept and β_i is an unknown parameter associated with the slope of the chemical i

[d] Where $\text{TU}_i = \frac{c_i}{\text{EC}_{x_i}}$. As can be shown, this approximation is equivalent to that of CA. See footnote a

[e] Where $\text{TEQ}_i = \frac{c_i}{\text{EC}_{50i}}$. As can be seen, TEQ method is a simplification of TU method where the slopes of the dose response curves of all the mixture components are considered identical, so that the contribution of each component to any effect level of the mixture is fixed as a proportion of the EC_{50}

[f] Where $E(c_{\text{mix}})$ is the total effect exerted by the total concentration of a mixture c_{mix}, $E(c_1)$ and $E(c_2)$ denote the fractional effects (x %) caused by the individual componenťs concentration c_1 and c_2. In the generalization, $E(c_i)$ is the fractional effects (x %) caused by the ith individual component

[g] Where $EC_{x,\text{mix}}$ is the total concentration of the mixture c_{mix} that produces a fractional effect x (x %). p_i is the molar fraction of the ith component in the mixture, EC_{xi} is the concentration of the ith that exerts a fractional effect x (x %) individually, and $E(c_i)$ is the fractional effect caused by the ith component of the mixture individually. α_{sim} is an *intra-similarity* parameter and α_{dis} is an *intra-dissimilarity* parameter. As can be seen, the INFCIM equation is a sum of the expected effects of the components under both CA and IA models but corrected with a parameter accounting for similarity and dissimilarity of the MOA of the components

[h] Where E is the effect is a function f of the parameter "c" which can be estimated by determining the value of four constants: a_1, a_2, b_1 and b_2. The value of the constants can be estimated from the information of the dose-response curves of the mixtures of the same components at different molar ratio mixtures (p_i)

also been applied in toxicology of mixtures (e.g., combination index-isobologram equation, fuzzy concentration addition-independent action, QSAR-based methods, statistical models).

We want also to describe some clarifying features of some of these other methodologies and their relationships with the CA and IA models. First, the additivity definition of CA model is the same than that of the classical isobologram [311, 318], the combination index (CI)-isobologram equation, and the statistical model developed by Gennings et al. [321]. The main difference in the case of the isobologram and CI with general CA model is that the additivity assumption in the biological response to the pollutant mixture is not made; therefore, both methods are actually oriented to find and quantify departures from additivity, such as synergism and antagonism [202, 312]. The method proposed by Gennings et al. [321] is a statistical theorem that analyses departures from additivity defined as the absence of changes in the slope of the chemicals in the presence of other chemicals (additivity criterion or no interaction). They demonstrated mathematically that their definition and the algebraic definition of additivity in CA model are totally equivalent [321]. Second, the methods to estimate departures from additivity usually used by authors working with toxic units are totally equivalent to the CA model. Toxic equivalency factor (TEQ)-based methods, on the other hand, are limited reformulations of the CA model that apply for a single level of effects, such as the one provided by the EC_{50}, and/or for mixtures of chemicals with identical dose response-curves of similarly acting chemicals [310]. The features of the rest of models summarized in the Table 3 will be explained if required when cited in the text.

The application of bioluminescent microorganisms to the issue of complex mixtures of pollutants/chemicals evolved in parallel to that of mixture toxicology. In some cases, the application of bioluminescent microorganisms to the mixture issue has helped in recent developments using other more ecologically relevant taxons, thereby contributing to the foundations of modern ecotoxicology of complex mixtures [310]. Pioneering studies in the application of bioluminescent microorganisms to assess the combined effect of complex mixtures can be found in the literature from the 1980s. Hermens et al. [246] applied the concept of CA to study the combined effect of organic chemicals on *V. fischeri* and linked their findings with results obtained for other ecologically relevant organisms, such as guppies, *D. magna,* and *Chlorella pyrenoidosa* [322, 323]. In the 1990s, Newman and McCloskey [324] developed a QSAR-based method to study the interaction between heavy metals using the toxicological information provided by the Microtox™ assay. They concluded that Cu interacted strongly with other metals ions (Ca, Cd, Hg, Mn, and Ni) and were able to model the toxicity of metals based on their constant of first hydrolysis in water.

Chen et al. [325, 326] performed a deep investigation of the interactions of binary mixtures of toxic reactive organic chemicals (such as acrylamide, formaldehyde, allyl alcohol, acetonitrile) using the Microtox™ assay. They applied the CA method designed solely for TU based on the EC_{50}, and an isobologram analysis also based on the EC_{50} values to study departures from additivity.

They found that synergistic effects were frequently observed, especially between chemicals with different MOAs. Some of the mixtures (malonitrile plus formaldehyde) were highly synergistic. Ince et al. [327] tested the interactions of binary mixtures of heavy metals using the Microtox™ test and the plant *lemna minor*. They developed a statistical method to check the significance of deviations from additivity and established a simple summation of toxic units as the additivity criterion. They found additivity and antagonism to be the predominant joint effects between heavy metals in both organisms.

At the end of the 1990s, Backhaus et al. [319, 328–331] started a new approach to the study of toxicology of mixtures, which led to the foundation of the basis of modern mixture toxicology and risk assessment [310]. They redefined the studies on drug mixtures by Berembaum [311, 317] (pharmacology) and applied them to environmental toxicology; they also studied the applicability of the additivity definitions of CA and IA in complex mixtures (more than ten components) of environmentally relevant pollutants (as herbicides and priority pollutants), focusing on low concentrations [319, 328–331].

The authors' former works where the initial hypothesis were launched were performed with the support of a bioluminescent microorganism (*V. fischeri*) [328–331], but their studies quickly focused on algae due to the ecologically relevance criterion [319, 320]. In these studies, they found that the toxicity of mixtures of similarly acting chemicals could be predicted by CA additivity [328], and that mixture of totally dissimilarly acting chemicals could be readily predicted by IA additivity [329]. They also proved that complex mixtures of individual pollutants present individually at concentration levels below their no-observed-effect concentrations could contribute effectively to the overall toxicity of the mixture, which was contrary to the mainstream opinion of the moment [310].

Early in the twenty-first century, genetically engineered bioluminescent microorganisms started to be applied to the study of the combined effect of pollutants. Preston et al. [121] applied the first group of genetically engineered microorganisms *E. coli* HB101 (pUC607) and *P. fluorescens* 10586 (pUC607) to study the combined effect of binary mixtures of heavy metals. As an additivity model, they applied and adapted a method proposed by Straton [332], which is basically a CA model (see Table 3). They found synergistic interactions between heavy metals and concluded that the mixture toxicity could not be modeled based on the individual metal toxicities. Strachan et al. [134, 135] studied the toxicological interactions of herbicides in soil matrixes also by using genetically engineered bioluminescent microorganisms (*E. coli* HB101, *P. fluorescens* 10586, *P. putida* F1, and Rhizotox-C). As an additivity test, they also used the method proposed by Straton [332]. They found a predominance of nonadditive responses (mainly antagonistic, but sometimes synergistic in mixtures of up to seven herbicides).

Mowat and Bundy [333] proposed a mathematical and computational methodology to assess the acute toxicity of chemical mixtures; they tested it with heavy metal combinations using the Microtox™ assay. In 2004, Utgickar et al. [334] explored the dependence of the individual and combined toxicity of Zn and Cu on

exposure time by using the Microtox™ assay. The definition of additivity they used is unclearly described in the chapter, but the results are quite interesting: they studied the enhancement of toxicity of each metal on the other as a function of time. For this, they prepared a solution of one of the metals and spiked it with a low concentration of the other. The authors found that both systems responded synergistically, but their temporal behavior was qualitatively different due to the differences in the ratio of the metals. They concluded that both mixture systems, although consisting of the same components, induced toxicity by a different mechanism of action.

During the 2000s, the application of CA and IA models inspired by the works of Backhaus et al. spread throughout ecotoxicology. With that, some limitations of these methods gained the attention of researchers—basically the requirement of previous knowledge on a mechanism of action in order to decide which method to use to estimate the toxicity of a mixture and their unsuitability to consider hormetic dose-response curves in the analysis. Furthermore, in the natural environment, chemicals mixtures of similar/dissimilarly acting chemicals will occur; consequently, neither method will predict toxicity accurately.

In 2004, Mwese et al. [335] developed a method called Integrated Fuzzy Concentration Addition–Independent Action Model (INFCIM; Table 3) to predict the combined toxicities of mixtures containing similar and dissimilarly acting chemicals. The method used molecular descriptors (QSAR) and the fuzzy set theory to group chemicals based on their degree of similarity prior to computing the model. The model proposed by Mwese is the first whole integration of the QSAR model's predictive capacity based on toxicological properties of individual chemicals and the predicted capacity of combined toxicology, so that the combined toxicity of similar and dissimilar acting chemicals could be predicted when acting jointly. The method was tested with several datasets and model organisms, including *V. fischeri* [335]. Its ability to predict the combined effect of mixtures of chemicals with similar and dissimilar MOAs was demonstrated.

At the same time that CA and IA additivity gained appreciation for the prediction of combined toxicity of complex mixtures and risk assessment [320, 336], some authors focused on mixtures of a reduced number of components, where the prevalence of additive behaviors was not so clear [283, 337–339]. Fulladosa et al. [282] found a general antagonistic interaction between binary mixtures of heavy metals towards *V. fischeri*, but a strong synergistic interaction in the case of Co and Cu combination. Ishaque et al. [338] investigated the joint toxicity of heavy metals in complex mixtures at environmentally relevant concentrations in soils (maximum contaminant levels allowed by the US EPA). They found a synergistic interaction in the complex mixtures applying a TU-based method (CA additivity) using Microtox™.

Tsiridis el al. [339] studied the combined toxicity of heavy metals and their modification by humic acids (HA) using *V. fischeri*. They found that, contrary to the expected results, the toxicity of heavy metals was not always alleviated by HA; that is, the toxicity of Pb was enhanced by HA. Cedergreen et al. [337] studied deviations from the additivity definitions of CA and IA induced by mixtures of low

number of components, especially focusing on pesticides (herbicides, insecticides, etc.). They tried to derive conclusions across organization/trophic levels in the ecosystems in vitro and in field conditions [340, 341]. For example, in their work: "Is prochlroraz a potent synergist across aquatic species? A study on bacteria, daphnia, algae and higher plants" [337], they studied the degree of uniformity of the response (synergism, additivity, or antagonism) of different organisms to mixtures of insecticides containing prochloraz (an ergostrol-biosynthesis inhibitor). As a representative organism of bacterial communities, they used *V. fischeri.*

From 2006 on, the study of combined toxicity using bioluminescent microorganisms came to a climax. There was an increasing number of published works that were more ambitious in their scope and applications. Dawson and coworkers [103, 342, 343] performed a series of basic mechanistic studies of binary mixtures of reactive and nonreactive chemicals (soft electrophiles and narcotics, respectively) on *V. fischeri*. They generated a series of well-designed experiments oriented to reveal the molecular site(s) of action of these chemicals. As measure of departures from additivity, they applied the methodology proposed by Gennings et al. [321] (see Table 3). Relevant findings of these studies are that the same chemicals can display their toxicity via more than one mechanism of action, and that these mechanisms of action can vary depending on the concentration of the chemicals [343].

Rodea-Palomares and coworkers [60, 201–203, 205, 344] applied the CI–isobologram equation [312, 318] to study the toxicological interactions of priority and emerging pollutants in aquatic organisms. This method is based on CA additivity, but it is not dependent on previous knowledge of the MOAs of the chemicals present in a mixture and is specifically oriented to study and quantify departures from additivity [312]. They tested heavy metals as well as emerging pollutants (lipid regulators, chlorinated pollutants, perfluorinated surfactants, and antibiotics) in binary and complex mixtures. As model organisms, they used a battery of aquatic organisms, including a natural bioluminescent organism (*V. fischeri*) and the recombinant bioluminescent cyanobacterium *Anabaena* CPB4337. Some of their most interesting findings are that the nature of the interaction between chemicals is strongly dependent on the effect level exerted by the mixture on the organism, so that the same pollutants can act synergistically at low effect levels and antagonistically at high effect levels [201]. They also found that emerging pollutants with a same pharmacological mode of action (as fibrates) can strongly interact synergistically in nontarget organisms by unknown toxicological modes of action [202], and that the nature of the interaction between chemicals is strongly dependent on the test species [203]. In a more recent study on the toxicity of complex mixtures of antibiotics [205], they found that classical CA and IA predictions underestimated the toxicity of antibiotics, and that these predictions could be improved by taking into account the information obtained by considering the CI as a correction factor (see Table 3).

At this point, the study of the toxicology of mixtures was mature enough to assess really complex systems and to address the problem of whole effluent toxicity from the viewpoint of the contribution of the individual components to the

overall toxicity. Parvez et al. [345, 346] tried to dissect the contribution of the different chemicals composing industrial mixtures to the overall toxicity and their level of interacting potential. For that purpose, they analyzed the chemical composition of the effluents of industries of main activities (such as organic chemicals, textile dye, pulp-paper, and petroleum refinery industries). They selected the main chemicals present in their effluents and studied their degree of interactions. They used CA and IA additivity definitions and developed a new experimental mixture design (2^n full factorial design) in order to be able to analyze mixtures with different number of components, different ratios, and different total concentration levels. They took advantage of a high-throughput configuration of the *V. fischeri* test. They identified some chemicals as naphtalene, *n*-butanol, *o*-xylene, catechol, and *p*-cresol as synergism-inducer chemicals [345, 346]. They found a prominent synergism in the mixtures corresponding to the pulp and paper industry and textile dyes [346].

Since 2010, studies on mixture toxicology have focused on validation of CA as a reference additivity hypothesis [347, 348], attempts to improving the CA model, and the search for the causes for synergism and antagonism [65–67, 349]. Liu and coworkers studied the mixture toxicity of pesticides, ionic liquids, phenols, and anilines, focusing on the improvement of mixture experimental design and models for improved evaluation of additivity and departures from additivity. They used *V. fischeri* and *V. quinghaiensis* sp.-Q67 as model organisms. Dou et al. [61] developed a new ray design (*EquRay*) to optimize the number and component of mixtures in order to maximize information minimizing the experimental work [61].

Liu and coworkers [290] developed new methods to overcome some of the limitations of CA and IA models. In 2010, they integrated hormesis in the CA by generating the mathematical methods to fit the hormetic dose-response curves, and they were able to predict the combined toxicity of complex mixtures of ionic liquids containing components with hormetic dose-response curves [290]. They also developed a method (called MM) for evaluating the toxicity of mixtures without requirement of the knowledge on the individual component toxicity (see Table 3). The method is based on an experimental correlation they found between a *location parameter* and the mixture ratio, so that, by assessing the experimental toxicity of mixtures of the same components at different ratios, they could define the mathematical function that describes the change of the toxicity of the mixture at the different ratios [349].

Zhang et al. [67] studied the chemical features of ionic liquids responsible for synergism and antagonism. They found that ionic liquids containing BF_4^- were responsible for the synergism/antagonism found; when they were removed from the complex mixtures, the deviations from additivity disappeared. Lin and coworkers [289, 350, 351] studied the factors influencing the joint effect of multicomponent mixtures by using *P. phosphoreum*. They found that, in general, the number of components in the mixture influenced the synergism/antagonism found in less complex mixtures of the same components. However, interestingly, they found that some *active* components can increase the toxicity of the complex mixtures if they interact with some components present in those mixtures [351].

These results agree with those found by Parvez et al. [346] (on synergism in model industrial waters) and Rodea-Palomares et al. [344], who found that perfluorinated surfactants (PFOS and PFOA) were able to increase synergism in complex mixtures containing priority and emerging pollutants.

Finally, some attempts to model the toxicity of chemicals in a mixture based purely on QSAR models have been proposed [251, 262, 352, 353], with limited repercussion in ecotoxicology. However, in recent years, there has been an increasing interest in generating mixed models integrating additivity predictions and QSAR modeling. For example, in the search for the causes of synergism, some authors are generating mixed models integrating additivity predictions and QSAR models [269, 289, 350, 351, 354]. Also, effect-based water-quality trigger values have been developed [172, 291], which are based on the use of QSAR models (to predict the individual expected toxicity of pollutants) at the level of individual component toxicity, and their expected mixture toxicities based on the additivity prediction of the CA and IA additivity models. Expected responses are grouped based on their theoretical modes of action via chemical motives computed also by QSAR, as proposed by Mwese et al. [335]. Interestingly, once again with the support of bioluminescent organisms (*V. fischeri*), the authors found that, when trying to accurately predict the toxicity of mixtures composed by the main pollutants of model wastewaters (up to 56 regulated chemicals), the selected chemicals were able to explain less than 1 % of the toxicity generated by the real wastewaters [291]. This finding, although disappointing, is the latest milestone in mixture toxicology. It opens up two possibilities that may be explored in the next future:

(1) Are the presently known and analytically identified chemicals just the tip of the mixture iceberg, such that we are actually unaware of the main unknown (not yet identified by chemical analysis) chemicals responsible for the toxicity found in the real natural environments?
(2) Or, is it that chemicals, when combined in a very complex scenario, result in interactions that we are unable to detect?

Bioluminescent microorganisms will surely play a pivotal role in the assessment of both possibilities.

6 Concluding Remarks

Biotoxicity tests based on bioluminescence microorganisms offer a sensitive, low-cost, easy-to-use, and quick methodology to detect potential toxicants to the whole environment. They complement analytical chemical techniques by focusing on bioavailability and cell toxicity. Genetic engineering has made it possible to create recombinant bioluminescent microorganisms that are more ecologically relevant and particularly useful in freshwater and terrestrial environmental matrices. Thus, the spectrum of bioluminescent microorganisms has increased substantially,

including both prokaryotes and eukaryotes, such as heterotrophic bacteria, cyanobacteria, yeast, fungi, and algae. This variety of bioluminescence bioassays allows the use of a battery of tests with organisms of different trophic levels, different sensitivities, and different response times, which will give complementary information on the toxicities of environmental samples.

Bioluminescence tests mostly focus on acute toxicity. However, bioluminescent assays with longer exposure times to pollutants have been developed. Commercial devices are available for many of the bioluminescent microorganisms, both natural and recombinant, allowing fully standardized methods that may be used in any laboratory around the world. Bioluminescent microorganisms have played a key role in the evolution of the concepts and methods to study the toxicity of complex mixtures of pollutants—a field that is fundamental to understand the real behavior of contaminants in the environment. The ease of maintenance and use of many bioluminescent microorganisms has made possible their immobilization and miniaturization in biosensor devices. In addition, whole-cell microarrays containing different bioluminescent cells (multitrophic test battery) allow simultaneous analyses of a range of pollutants and environmental samples. They may further be configured for continuous, online, and in situ environmental monitoring.

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Molecular Manipulations for Enhancing Luminescent Bioreporters Performance in the Detection of Toxic Chemicals

Sharon Yagur-Kroll and Shimshon Belkin

Abstract Microbial whole-cell bioreporters are genetically modified microorganisms that produce a quantifiable output in response to the presence of toxic chemicals or other stress factors. These bioreporters harbor a genetic fusion between a sensing element (usually a gene regulatory element responsive to the target) and a reporter element, the product of which may be quantitatively monitored either by its presence or by its activity. In this chapter we review genetic manipulations undertaken in order to improve bioluminescent bioreporter performance by increasing luminescent output, lowering the limit of detection, and shortening the response time. We describe molecular manipulations applied to all aspects of whole-cell bioreporters: the host strain, the expression system, the sensing element, and the reporter element.

Keywords Bioreporters · Biosensors · Bioluminescence · Fluorescence · *Escherichia coli* · Genetic engineering

Contents

1 Introduction 138
2 Genetic Manipulations of the Host Strain and Expression System 139
3 Genetic Manipulations of the Sensing Element 140
4 Genetic Manipulations of *lux* Reporting Elements 143
5 Additional Genetic Manipulations and the Advent of Synthetic Biology 144
6 Summary 147
References 148

S. Yagur-Kroll · S. Belkin (✉)
Institute of Life Sciences, The Hebrew University of Jerusalem,
91904 Jerusalem, Israel
e-mail: shimshon.belkin@mail.huji.ac.il

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1 Introduction

Analytical chemical and physical methodologies for monitoring environmental pollution can be powerful, accurate, and sensitive, but they fail to provide data on the bioavailability of pollutants or their effects on living systems. Whole-cell-based bioassays offer a complementary approach, by reporting not only on the presence of a chemical but also on its biological effects. Such bioreporters, in most cases, are genetically modified microorganisms that produce a dose-dependent quantifiable output in response to the presence of toxic chemicals or other stress factors.

In general, genetically engineered microbial bioreporters combine a sensing element (usually a gene regulatory element responsive to the target) and a reporter element, the product of which is quantitatively monitored either by its presence or by its activity. Commonly used reporter genes include *lacZ,* the product of which may produce (depending upon the substrate used) a chemiluminescent, colorimetric, electrochemical, or fluorescent signal, and diverse fluorescent protein genes such as *gfp* [1–3]. This chapter focuses on another popular reporting option, bioluminescence, based either on the bacterial *luxCDABE* genes or on the firefly luciferase *luc* gene.

The *luxAB* genes encode the α and β subunits of the luciferase dimer, which catalyzes the oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde in the presence of molecular oxygen, culminating in the emission of blue-green 490-nm light. The synthesis of the aldehyde is catalyzed by a fatty acid reductase complex that includes a reductase, a transferase, and a synthetase, coded for by the *luxC*, *D*, and *E* genes, respectively [4]. A *lux*-based luminescent bioreporter can be based either on the complete *luxCDABE* cassette, enabling substrate-independent real-time activity monitoring, or on the luciferase genes (*luxAB*) only. In the latter case, an external addition of a substrate (usually decanal or nonanal) is required, and activity is measured at a single time point rather than continuously. The firefly Luc protein catalyzes the oxidation of a reduced luciferin in the presence of ATP-Mg^{2+} and oxygen to generate a yellow-green 562-nm light signal, the quantum yield of which is the highest of any of the currently characterized bioluminescent systems [3]. The Luc protein requires no post-translational modifications and is thus immediately active upon translation. As for the bacterial *luxAB* option, *luc* reporter systems require the exogenous addition of a luciferin substrate.

The first bioluminescent bacterial sensor, a *Pseudomonas* strain engineered to detect naphthalene, was reported in 1990 [5]. Since then, numerous reports have described the construction of sensor cells capable of reporting the presence of either specific chemicals, classes of compounds, or global stress factors, such as toxicity or genotoxicity [1, 3, 6]. In this chapter, we focus on molecular manipulations applied to luminescence bioreporters in order to enhance their performance by rendering their responses faster, stronger, and/or of a lower detection threshold. We differentiate between reporter-independent manipulations of a general nature applied either to the bacterial host or to the sensing element, and

luminescence-specific modifications. We also describe potentially useful genetic manipulations that can be easily applied to improve performance of luminescent reporters, although they have been originally reported in relation to different molecular constructs.

2 Genetic Manipulations of the Host Strain and Expression System

It has been demonstrated on numerous occasions that the same promoter-reporter fusion may be expressed to a different extent when carried by different bacterial hosts. Hynninen et al. [7] reported an improvement in the detection limit for heavy metals by *Pseudomonas putida*-based Cd/Zn/Pb-bioreporters, harboring fusions of the heavy-metal-regulated *cadA1* and *czcCBA1* gene promoters to *luxCDABE* in a low copy number plasmid, by altering metal efflux from the cytoplasm. This has been achieved by disrupting four metal efflux transporter genes (P-type ATPases CadA1 and CadA2, and CBA transporters CzcCBA1 and CzcCBA2), thereby causing intracellular accumulation of the metals and thus enhancing cellular sensitivity. The *czc*-based system, when expressed in the metal transporter-deficient mutant, detected Pb^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+} at concentrations 45-, 12-, 10-, and 3-fold, respectively, lower than its wild-type counterpart. Similarly, the Zn detection threshold by the *cadA*-based system was approximately 50 times lower than that possible with other available Zn bioreporters. The improved detection limits were significantly below the regulatory levels for Zn and Pb in water and in soil.

Modifying the host cell's toxicant efflux capacity of *Escherichia coli* by a *tolC* mutation, which limits the ability to pump out undesired molecules [8], lowered the detection threshold of a sensor strain harboring a fusion between heat shock promoter *grpE* to *lux* genes, to the hydrophobic pentachlorophenol [9] and of a sensor strain harboring a fusion between SOS promoter *recA* to *lux* genes to genotoxicants [10]. Davidov et al. [10] also compared the performance of the same *recA*::*lux* plasmid in *E. coli* and in *Salmonella typhimurium*; the latter is attractive for its established role in the Ames reverse mutation assay [11]. They reported a much faster response in *Salmonella*, as well as an increased sensitivity to hydrogen peroxide, but not to mitomycin C (MMC) and 1-methyl-3-nitro-1-nitrosoguanidine.

The use of bacterial reporters for the detection of antibiotics is complicated by the fact that antibiotic resistance genes are normally employed for selection and plasmid maintenance, and the detection limits are thus strongly influenced by the antibiotic's innate toxicity to the reporter organism. A way to overcome this problem was reported by Melamed et al. [12], who described a nonantibiotic selection system based on the requirement for tryptophan. A tryptophan auxotroph *E. coli* mutant (Δ*trpE*) was used as a host strain; a plasmid that lacks antibiotic resistance genes, but confers the ability to produce tryptophan, was used as the parental vector in which the sensing element was fused to *luxCDABE*.

Reporter performance can also be dramatically affected by the number of sensing-reporting modules in the reporter cell, ranging from a single chromosomal integration to multiple copies in plasmids of diverse copy numbers. A multicopy plasmid [13] may enhance the intensity of the observed response, but it introduces a potential instability into the maintenance of the extrachromosomal genetic element. Another possible disadvantage of a multicopy plasmid-based fusion is a loss of responsiveness to the regulatory element due to a titration effect of hundreds of fusions containing operator-promoters on a fixed level of regulatory molecules. When the performance of a chromosomally integrated single copy of the same *recA*::*lux* segment was compared to a plasmid-borne multicopy fusion [10], the single-copy chromosomal integration led to a longer lag period, lower luminescence levels (in both noninduced and induced states), increased response ratios, and enhanced sensitivities. The two latter points were direct consequences of a decreased background luminescence.

3 Genetic Manipulations of the Sensing Element

In most engineered bioreporters, the sensing element is a promoter region that controls the expression of the reporter gene(s), but the extent to which the promoter-containing segment should be extended downstream into the open reading frame (ORF) has rarely been addressed. Yagur-Kroll et al. [14] fused two gene promoters (*sulA,* induced by SOS-activating genotoxicants, and *grpE,* induced by diverse stress conditions) to *luxCDABE* in two versions: a short one that terminated upstream of the gene and a long one that extended for about 200 bp into its ORF. Surprisingly, different results were obtained for the two tested promoters. In the case of *sulA*, the longer version exhibited a significant advantage over the shorter, as it responded to lower inducer concentrations, produced higher luminescence values, and generated higher response ratios. The opposite phenomenon was observed in the case of *grpE,* in which the short version was advantageous to the longer one. Thus, while a modification of the sensing element length can have a significant influence on bioreporter performance, the effect can be gene-specific and may need to be individually optimized.

Another tactic to improve bioreporter performance is by duplicating different elements in the sensing region, in an attempt to enhance their effect. A dual-promoter bioreporter was constructed by inserting the sequence of the *recA* promoter between *sulA* and *luxCDABE* genes, generating a *sulA*::*recA*::*luxCDABE* construct [14]. Because both *sulA* and *recA* are induced by genotoxic agents, bioluminescence intensity was much higher for the dual-promoter than that of either of the single-promoter fusions (*recA* or *sulA*). The lowest detected concentration of nalidixic acid by the dual-promoter sensor was half of that of the *sulA* construct, but similar to that of *recA* alone. In another report, a binding site for ArsR, a regulatory protein that controls the basal level of gene expression of the *ars* operon, was duplicated in an *E. coli ars*-based arsenic bioreporter. This

duplication was optimized to reduce background expression in the absence of arsenic [15].

Changing specific nucleotides in specific positions, using site-directed techniques, has also proven to be efficient in improving bioreporter abilities. A *S. typhimurium* bioluminescent genotoxicity tester strain (VITOTOX®) was constructed [16], harboring a fusion of the *E. coli recN* promoter to the *Aliivibrio fischeri luxCDABE* cassette. A faster and stronger induction was observed with a mutant containing a G nucleotide at the consensus position of the promoter −35 region compared to the wild-type, probably due to a better binding of the RNA-polymerase to the mutated σ^{70}-like promoter sequence. Yagur-Kroll et al. [14] changed the −35 element of the *sulA* promoter in a *sulA*::*luxCDABE* fusion to the consensus sequence, based on the report that this sequence provides the strongest promoter activity [17]. Indeed, a much faster and stronger induction, accompanied by an improvement of detection sensitivity, was achieved. In this regard, it is worth noting that when the −10 consensus sequence was changed into a combination that took into account the multiple pathway of transcription initiation [18], induction by SOS-activating agents was completely abolished.

An opposite approach to the precisely planned point mutations in specific positions is random mutagenesis in a "directed evolution" process. The approach is generally based on error-prone polymerase chain reaction (PCR), with or without the combination of a 'DNA shuffling' procedure, that may be performed on the target DNA sequence; the resulting library of variants is then screened for the desired feature and selected isolates are reprocessed in the same manner. This procedure is often applied for modifying protein sequence and performance, but it may just as well be applied to regulatory areas of the bacterial genome. In one cycle of an error-prone PCR performed on the *sulA* promoter in a *sulA*::*luxCDABE* reporter, an improved mutant was isolated, harboring three point mutations relative to the wild-type sequence [14]. This mutant exhibited a much faster and stronger response to a genotoxic agent (nalidixic acid) and lower detection thresholds compared with the wild type. When each of the three point mutations was individually introduced into the wild-type promoter, it was found that the effect was mostly mediated by an A to G substitution in close proximity to the consensus LexA repressor box. The fact that performance was improved, even though the mutations were not located within well-defined regulatory sequences, demonstrates the potential power of the 'directed evolution' process, which introduces mutations into random locations that would not necessarily be identified as obvious targets by bioinformatic tools.

This approach has particular potential in cases when the sensing element is a promoter of an uncharacterized gene, as in the case of a 2,4 dinitrotuloene (2,4-DNT) bioreporter [19], based on the *yqiF* gene promoter. Performance of the *yqjF*::*luxCDABE* fusion was significantly improved by two rounds of directed evolution. The variant isolated in the second round exhibited a much stronger bioluminescent response; detection time was shorter (40 min as compared to 60 min for 78 mg L^{-1} 2,4-DNT), and the detection threshold was 4-fold lower. The improved variant had four point mutations compared to the wild type, none of

which was located in a predicted regulatory element in the *yqjF* promoter (−10, −35 or Shine-Delgarno (SD) elements), once again emphasizing the inherent power of the "directed evolution" tool.

A *Pseudomonas* bioreporter for toluene and related compounds, based upon the *xylR* regulatory gene of the TOL plasmid, was subjected to improvements using several different approaches. In this sensor, toluene activates the regulatory protein XylR that positively controls the *Pu* promoter, resulting in bioluminescence from the *Pu*::*luc* fusion. The ability of two distinct nucleotide sequences to function as SD elements and improve sensitivity of bioreporting was evaluated [20]; it was found that one (taaggagg) was far more effective than the other (aggaaa) in inducing reporter gene expression. A terminator placed in-frame and upstream of the *Pu* fragment minimized background expression of the *luc* gene, but also moderately reduced the intensity of the response. These results highlight the importance of the selection of the most suitable genetic elements, such as SD and terminator sequences, in optimizing the specificity and sensitivity of bioreporter plasmids.

The same XylR regulator was employed to detect nitrotoluenes, predominant land mine components [21]. Two experimental strategies were employed to generate combinatorial XylR libraries, produced either by shuffling DNA segments of XylR with those of the homologous N-terminal domain of the phenol-responding regulator DmpR, or by random introduction of single amino-acid changes by error-prone PCR. One variant (XylR5) generated a better signal-to-noise ratio in response to 2,4 DNT; it was further fused to *luxAB* and used in pilot experiments for detecting this compound in soil. The latter approach is an example of reporter enhancement by modification of regulatory protein sequences that alters protein properties, as opposed to manipulations of regulatory noncoding sequences.

In the same system, an innovative way to enhance *xylR* activity and improve its specificity was reported by de las Heras et al. [22], who altered the working regimes of the relevant genetic circuits by simple changes in the way upstream transcription factors are self-regulated by positive or negative feedback loops, rather than by modifying XylR structure. The natural negative feedback loop regulating *xylR* transcription was modified with a translational attenuator that lowered the response to 3-methylbenzylalcohol (3MBA) while maintaining the transcriptional output induced by *m*-xylene. XylR expression was then subject to a positive feedback loop in which the TF *xylR* was transcribed from its own target promoters. In the first case (*xylR* under the strong promoter of the upper TOL operon, *Pu*), the reporter system displayed an increased transcriptional capacity for both optimal and suboptimal XylR effectors. In contrast, when *xylR* was expressed under a weaker promoter (the XylS promoter *Ps*), the resulting circuit accurately discriminated *m*-xylene from 3MBA. The engineered connectivity yielded higher promoter activity as well as an increased signal-to-background ratio and enhanced specificity.

Another example of improvement by altering protein abilities is of NahR, a member of the LysR regulator family, a positive transcriptional regulator for genes of the naphthalene degradation pathway in *Pseudomonas* [23]. Five single and six

double mutations were introduced at residues 169 and/or 248, located in the central inducer recognition domain and the C-terminal multimerization domain of the protein, respectively. The effects of these mutations were examined by monitoring the expression of a *luc* reporter gene under the control of NahR. In contrast to the wild-type NahR that responded only to salicylate, all mutants responded to induction by both salicylate and benzoate. To further improve sensitivity, additional mutations were introduced in residues 169 and 248 [24], displaying a significantly lowered (up to 50-fold) detection thresholds.

In another study, a *Pseudomonas* phenol bioreporter based on DmpR (dimethyl phenol regulatory protein), an NtrC-like regulatory protein for phenol degradation, was constructed. DmpR was expressed from its promoter, Pr, a σ_{70}-dependent promoter regulated by the σ_{54} dependent promoter, Po, fused to *luc*. DmpR was selected because its natural interaction with several phenol derivatives suggested that a modification of its sensor domain might result in a protein that can detect a broader range of phenols [25]. Computationally predicated residues were mutated, significantly altering effector specificity. A mutant with the single amino acid transposition F42L exhibited superior luciferase activity compared with the wild-type, as well as an enhanced sensitivity to various phenol derivatives, including the highly toxic and possible carcinogen 2,4-dimethylphenol.

4 Genetic Manipulations of *lux* Reporting Elements

Bioluminescence genes from diverse sources, both prokaryotic and eukaryotic, may be employed as reporter elements in genetically engineered microbial bioreporters; the specific characteristics of each should be taken into consideration. For example, one of the problems often accompanying the use of *E. coli* as a luminescent reporter is the necessary compromise in working temperature: the optimal expression temperature of the commonly used *A. fischeri* luciferase is significantly lower than the 37 °C required for "normal" *E. coli* functions. One possible solution is the use of the *luxCDABE* cassette of *Photorhabdus luminescens* [26], the luciferase of which operates readily at 37 °C. When performance of the *P. luminescens* luciferase in *E. coli* was compared to that of *A. fischeri*, Davidov et al. [10] have shown that the former allowed a significant shortening of response time.

The *P. luminescens luxCDABE* cassette was also instrumental in a demonstration of an improvement of bioreporter performance by rearranging the *luxCDABE* genes and splitting them into two independent functional units: *luxAB*, coding for the luciferase enzyme, and *luxCDE*, coding for the reductase complex [27]. The hypothesis that splitting the five-genes operon will be beneficial was driven by three main assumptions:

(1) Transcription and translation of a short operon should be faster, more effective, and more efficient than that of a longer one.

(2) By a high and constant expression of one component of the bioluminescence reaction, either *luxAB* or *luxCDE*, only the complementary component should be induced, thus leading to a faster and/or stronger response.
(3) Constant expression of *luxCDE* genes may overcome aldehyde limitation.

This was tested by placing each of the two units under the regulatory control of either an inducible promoter or under a moderate constitutive promoter (*CP38*). The two units were co-expressed in *E. coli* and were tested for their ability to be induced by a model chemical in comparison to the original *luxCDABE* system. In all cases, the combination of a constitutively expressed *luxCDE* with an inducible *luxAB* resulted in dramatically stronger and faster responses to all tested target chemicals. This is demonstrated in Fig. 1 for the *sulA*-based genotoxicity reporter induced by three DNA damaging agents. All other split combinations also appeared to be superior, to various extents, to the native *luxCDABE* combination, displaying enhanced sensitivities and higher response ratios.

The *luxCDABE* operon of *P. luminescens* is relatively rich in codons, having either A or T in the wobble position (69 %), suggesting that these genes would not be efficiently expressed in high-GC organisms such as *Streptomyces*. As a solution to this problem, an optimized synthetic *luxCDABE* operon encoding the *P. luminescens* Lux proteins that lacks TTA codons and in which the majority of codons end in a G or C was constructed for efficient expression in high-GC bacteria [28]. Another modification of the *P. luminescens lux* system addressed the decay constants of reporter proteins; a relatively long half-life of the reporter protein(s) prevents monitoring both the initiation and the termination of transcription in real-time and may significantly degrade circuit performance. Short half-life variants of LuxA and LuxB were constructed in *E. coli* by inclusion of an 11-amino acid carboxy-terminal tag that is recognized by endogenous tail-specific proteases, thus accelerating the degradation of these proteins [29]. Addition of the C-terminal tag reduced the functional half-life of the holoenzyme by approxiately 5-fold when the tag was added to *luxA* or to both *luxA* and *luxB*, but not to *luxB* alone. It was also found that alteration of the terminal three amino acid residues of the carboxy-terminal tag fused to LuxA generated variants with half-lives of intermediate lengths.

5 Additional Genetic Manipulations and the Advent of Synthetic Biology

Although the title and main scope of this chapter refers to bioluminescent bioreporters, in accordance with the overall theme of this book, a few reported molecular manipulations of nonbioluminescent bacterial sensors are worth mentioning because the same principles can easily be applied irrespective of the reporter function.

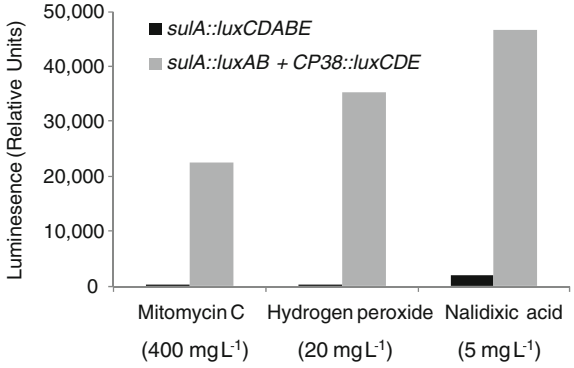

Fig. 1 Bioluminescence induction in response to genotoxicants in a "complete" *sulA*::*luxCDABE* reporter compared to a split system, in which the *luxAB* genes are inducible and the *luxCDE* genes are constitutively expressed (*sulA*::*luxAB* + *CP38*::*luxCDE*)

The promoter/operator of the *ars* operon (P_{ars}) and the *arsR* gene of *E. coli* are parts of a *cis*-active negative feedback loop that is downregulated by ArsR and has been applied to the transcriptional switches of the reporter gene in arsenic bioreporters. In an attempt to improve the signal-to-noise ratio and the detection limit of these sensors, recombinant *E. coli* cells were transformed with two plasmids, in which P_{ars} and *arsR* are placed in *trans*: one harboring three tandem copies of the *ars* promoter/operator fused to the *gfp* gene, and the other harboring one copy of IPTG (isopropyl-1-thio-β-D-galactopyranoside)-controlled *arsR* gene [30]. This manipulation doubled the signal-to-noise ratio and lowered the As(III) detection limit from 20 to 7.5 μg L^{-1}, compared to the use of a plasmid harboring one copy of the *ars* promoter/operator-*arsR*-*gfp*. In a different system, duplication of a promoter::reporter fusion yielded variable results. Detection of 2,4-DNT by a *ybiJ*::*gfpmut2* fusion [31] was significantly affected by the introduction of an additional copy of the fusion, but the effect depended on whether the two copies were present on the same plasmid or not (Fig. 2). The two-plasmid combination appeared to be preferable at lower concentrations, whereas the opposite appeared to be true at the higher concentration range.

An *E. coli* phenol bioreporter harboring the phenol-inducible *mphK* promoter from *Acinetobacter calcoaceticus* fused to a *lacZ* reporter gene, as well as the regulator gene mopR, was reported by Peng et al. [32]. Phenol detection sensitivity (0.1–5 μM) was improved by 2-fold following the deletion of one out of three inverted repeats (IR1) located upstream of the σ^{54}-dependent promoter of *mphK*.

Microbial engineering often requires fine control over protein expression. To avoid the need for trial-and-error optimization of an engineered genetic circuit, Salis et al. [33] developed a predictive design method that takes into account the DNA sequence of ribosome binding sites (RBSs) and their function inside a genetic system—that is, controlling translation initiation rate and protein expression level. The prediction combined a biophysical model of translation initiation with an optimization algorithm to predict a synthetic RBS sequence that provides a

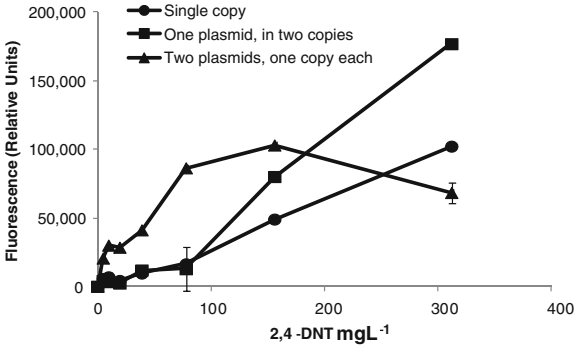

Fig. 2 Dose-dependent fluorescence response of a 2,4-DNT-sensing reporter strain: Comparison of a single sensor::reporter module (*ybiJ*::*gfp)* to two copies of the same module, expressed either from one plasmid or from two compatible plasmids

target translation initiation rate on a proportional scale. This prediction method can be used to improve translation initiation of a bioreporter, often the rate-limiting step in bacterial translation, thereby further improving biosensor performances.

To date, there are only few examples of the application of synthetic biology tools to enhance bioreporter performance. One such modular design strategy was used by Kobayashi et al. [34] to develop *E. coli* bioreporters that respond to signals in a programmable fashion. The modular design strategy is based on a genetic toggle switch that flips from an *off* to an *on* state when a signal threshold is exceeded, combined with SOS network elements as the sensor module. The switch is comprised of two genes, *lacI* and λ *cI*, which encode the transcriptional regulator proteins LacR and λ CI, respectively (Fig. 3a). The *lacI* gene is expressed from a modified P_L promoter, P_L^*, which is repressed by λ CI; the λ *cI* gene is expressed from another promoter, P_{trc}, which is repressed by LacR. The RecA coprotease is activated in the presence of single-stranded DNA, leading to λ CI degradation, resulting in derepression of the P_L promoter and of *gfp* expression. Treatment with 1 ng mL^{-1} and 10 ng mL^{-1} MMC yielded a 1.9- and 19-fold increase in the population-averaged fluorescence signal, respectively, representing a significant improvement in the limit of detection. Comparing this strain with a control that lacks the *lacI* feedback gene demonstrated that the feedback architecture of the genetic toggle switch provided at least a 1,000-fold improvement in sensitivity and enabled the readout of a detection event long after the DNA-damaging agent was removed.

A different toggle switch was used to improve the performance of a *P. putida* Cd(II) bioreporter [35]. The gene circuit contained the *cadR* (Cd-responsive regulator gene) promoter (P_{cadR}), regulating the $lacI^q$ and *gfp* genes, and the *tac* promoter (P_{tac}), regulating the *cadR* gene (Fig. 3b). When cadmium is present, P_{cadR} is induced to produce LacI and GFP. LacI represses P_{tac}, and GFP expression is used to monitor the level of P_{cadR} induction. When IPTG is added, P_{tac} induces production of CadR, which in turn represses P_{cadR}, and dampens GFP production. When this circuit was induced by cadmium, the toggle biosensor exhibited a lower background, a higher signal-to-noise ratio, and an improved sensitivity when compared with two other non-toggle gene circuits. This improved biosensor was highly specific to cadmium (II) and did not respond to other valence II cations.

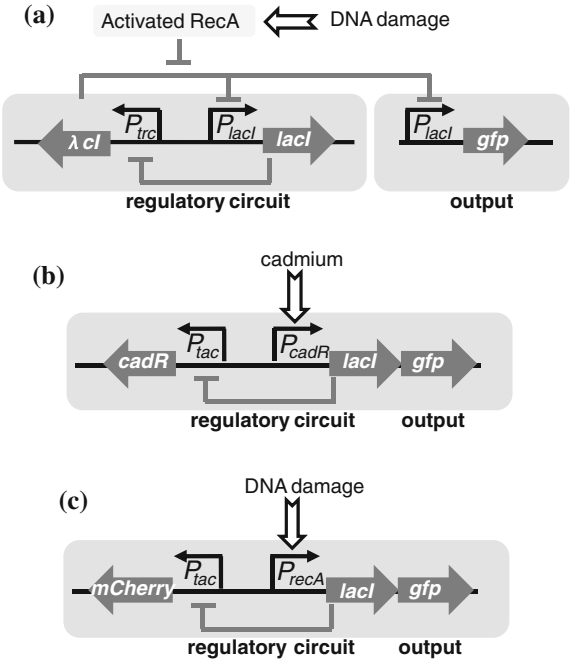

Fig. 3 Examples of engineered gene circuit diagrams of microbial reporters. **a** A genotoxicant-responsive reporter based on interfacing the genetic toggle switch with the SOS network. **b** A cadmium-responsive reporter based on interfacing a genetic toggle switch with the Cd-responsive regulator gene. **c** A genotoxicant-responsive reporter based dual-reporter cascade. Modified from [34–36]

A synthetic regulatory construct based on a two-stage amplifying promoter cascade was applied to whole-cell biosensing of DNA-damaging agents, based on the *E. coli recA* and *tac* gene promoters, fused to green (GFP) and red (*mCherry*, RFP) fluorescent protein genes, respectively [36]. The two promoters were linked by the LacI repressor in *E. coli*, where DNA-damage activates the *recA* promoter and hence the upregulation of GFP and LacI (Fig. 3c). LacI, in turn, represses the *tac* promoter, downregulating the otherwise constitutive *mCherry* transcription. Thus, the absence of DNA damage results in a low expression of GFP and a high expression of RFP. Conversely, sensed DNA damage drives a high level of GFP expression and a low level of RFP expression. The detection threshold of MMC by this dual-reporter construct was as low as 0.1 nM, compared to 0.25 and 2.0 nM by the single-ended reporters *recA*::*mCherry* and *recA*::*gfpmut3.1*, respectively.

6 Summary

In this chapter we have attempted to summarize diverse molecular manipulation approaches reported to improve bioreporter performance in terms of signal intensity, response times, and target detection thresholds. Although many of the undertaken molecular engineering directions yielded positive results, the field is still wide open for additional improvements. Three relatively recent developments

currently promise to be highly rewarding: the increased availability of high-throughput automation, the rapidly developing field of synthetic biology, and the use of bioinformatics and in silico design. Singly or in combination, these approaches can help target the genetic element to be modified, outline the preferred modifications to be introduced, and provide the technical ability to do so. It should also be born in mind that, in some cases, the approaches to be undertaken need to be sensor-specific, and that an "unbiased" high-throughput trial-and-error strategy may sometimes prove to be just as effective as a carefully designed genetic manipulation.

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Part III
Applications of Bioluminescence in Agriculture and Bioprocess

Biosensors, Antibiotics and Food

Nina Virolainen and Matti Karp

Abstract Antibiotics are medicine's leading asset for fighting microbial infection, which is one of the leading causes of death worldwide. However, the misuse of antibiotics has led to the rapid spread of antibiotic resistance among bacteria and the development of multiple resistant pathogens. Therefore, antibiotics are rapidly losing their antimicrobial value. The use of antibiotics in food production animals is strictly controlled by the European Union (EU). Veterinary use is regulated to prevent the spread of resistance. EU legislation establishes maximum residue limits for veterinary medicinal products in foodstuffs of animal origin and enforces the establishment and execution of national monitoring plans. Among samples selected for monitoring, suspected noncompliant samples are screened and then subjected to confirmatory analysis to establish the identity and concentration of the contaminant. Screening methods for antibiotic residues are typically based on microbiological growth inhibition, whereas physico-chemical methods are used for confirmatory analysis. This chapter discusses biosensors, especially whole-cell based biosensors, as emerging screening methods for antibiotic residues. Whole-cell biosensors can offer highly sensitive and specific detection of residues. Applications demonstrating quantitative analysis and specific analyte identification further improve their potential as screening methods.

Keywords Antibiotic residue · Confirmatory method · Food safety · Screening method · Whole-cell biosensor

N. Virolainen · M. Karp (✉)
Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland
e-mail: matti.karp@tut.fi

N. Virolainen
Ductor Corporation, Helsinki, Finland

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Abbreviations

CCα	Decision limit
CCβ	Detection capability
CPMA	Combined plate microbial assay
EU4pt	EU fourplate test
HPLC	Highperformance liquid chromatography
IF	Induction factor
IP	Identification point
LC	Liquid chromatography
MLS	Macrolides, lincosamides and streptogramins
MRL	Maximum residue limit
MS	Mass spectrometry
MRSA	Meticillin-resistant *Staphylococcus aureus*
NAT	Nouws antibiotic test
SPR	Surface plasmon resonance
STAR	Screening test for antibiotic residues
TC	Tetracyclines
WHO	World Health Organization

Contents

1 Introduction 154
2 Antibiotic Use and Associated Risks 155
3 Control of Antibiotic Use 157
4 Detection Methods of Antibiotic Residues in Food 159
4.1 Confirmatory Analysis of Antibiotic Residues 159
4.2 Screening Methods for Antibiotic Residues 161
5 Biosensors for Detection of Antibiotics in Food 167
5.1 Whole-Cell Biosensors 171
5.2 Inducible Whole-Cell Biosensors for Antibiotic Detection 173
6 Conclusions 179
References 179

1 Introduction

The widespread and often excessive use of antibiotics in health care and agriculture has led to the appearance of resistant pathogens. This is surmised to lead to an emergence of novel perilous infections and a revival of diseases that were already considered to be eradicated. Antibiotic resistance is a common phenomenon that has evolved simultaneously with the capacity to produce antibiotics, dating back millions of years [6]. However, due to human activity, the number of resistant organisms has risen above normal in recent decades [112]. The problem

of increasing resistance has been taken into consideration by international organizations and governments, which have devised guidelines for antibiotic stewardship in both veterinary and human medicine.

European Union (EU) legislation enforces the establishment of national monitoring plans, under which a set percentage of animal products is monitored for antibiotic and other residues using screening and confirmatory methods of predetermined quality [42, 46]. Microbial growth inhibition tests are currently the most commonly used screening method for antibiotic residues in food. These methods, however, have sensitivity and specificity problems that can lead to false negative results.

This chapter presents an overview of the methods currently used in the EU for screening and confirmatory analysis of antibiotic residues in food and discusses the use of biosensors as a novel screening and confirmatory method. The use of inducible whole-cell biosensors as a screening method is considered. These biosensors are living bacterial cells that have been genetically engineered to produce a signal in the presence of the analyte, the antibiotic molecule. Whole-cell biosensors are an affordable screening method that can offer sensitive and specific analyte recognition.

2 Antibiotic Use and Associated Risks

Antibiotics are one of the most well-known and well-used groups of antimicrobial agents and the number-one asset in medicine for fighting microbial infection. The busiest time in antibiotic discovery was after World War II when, within 10 years, most of the antibiotic classes still in clinical use were discovered. Antibiotics, along with vaccinations and improved sanitation, have contributed significantly to the control of infectious diseases that were once among the leading causes of human morbidity and mortality [25]. Veterinary antibiotic use has contributed to improvements in animal health and welfare and to a marked increase in productivity of livestock for human consumption [56].

In animal husbandry, antibiotics are used for disease therapy, disease control, and growth promotion. Disease control refers to prophylactic treatment of all animals in a group when one or more group members show signs of disease [75]. Growth promotion with subtherapeutic doses of antibiotics increases viability and the rate of weight gain, as well as reduces the amount of feed per unit of gain [39, 75].

The antibiotics market amounted to global sales of US$25 billion in 2005 and US$42 billion in 2009, representing 5 % of the global pharmaceutical market and 46 % of sales of all anti-infective agents [81]. The total global use of antibiotics is estimated to be between 100,000 and 200,000 tons per year [120], including antibiotics used in human and veterinary medicine and as growth promoters. Veterinary antibiotics make up approximately one-third of total antibiotic sales in the EU [120].

Table 1 Antibiotic use in the EU and Switzerland in metric tons

Year	Human medicine	Veterinary medicine	Growth promotion	Total	Ref.
1997	7,659 t (60 %)	3,494 t (27 %)	1,599 t (13 %)	12,752 t (100 %)	[56, 177]
1999	8,528 t (65 %)	3,502 t (29 %)	786 t (6 %)	13,216 t (100 %)	[120, 177]
2004	n.r.	5,393 t[a]	n.r.	n.r.	[98]

n.r. = not reported
[a] Estimated use in the EU

The use of veterinary medicinal antibiotics in the EU increased by 54 % between the years 1997 and 2004 (Table 1). The increase mostly resulted from the gradually enforced (from 1999 to 2006) ban on using antibiotics for growth promotion, which led to an increase in the use of therapeutic antibiotics [24, 166]. However, the increase in veterinary use leveled off between the years 2005 and 2009 [55].

Antibiotics are used excessively and with little attention to the inevitable consequence of resistance [40]. Antibiotics not only act on pathogenic bacteria but also on a myriad of commensal bacteria, creating a reservoir of resistant organisms [22, 199]. The first reports of resistance appeared shortly after the therapeutic use of antibiotics commenced in the 1940s [3, 118, 188]. This was inevitable because all bacteria harbor some degree of antibiotic resistance due to nonspecific efflux systems, and most bacteria also have genes for more specific resistance [6, 37, 199]. In addition, bacteria can acquire resistance mechanisms through horizontal gene transfer [128, 153]. Multiresistant strains harboring co-selected multiple resistance genes are a result of a recent evolution process intensified by human activities [37, 57, 128].

The increase in the number, diversity, and range of resistant organisms has become an enormous clinical problem in the form of superbugs such as meticillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci [55, 153, 199]. Hospital-acquired infections caused by multiply-resistant bacteria affect approximately 7 % of patients (more than 4 million) in the EU annually [52]. Approximately 37,000 deaths are directly caused by hospital-acquired infections, and they contribute to an additional 111,000 deaths. The total annual healthcare cost of nosocomial infections in the EU is estimated at €7 billion.

Because antibiotics are used in both human and veterinary medicine, a pool of antibiotic resistance has emerged that has the potential to spread between animals and humans. Antibiotics are misused in human medicine through self-medication, over-the-counter availability, and needless prescriptions to treat viral infections [40]. Misuse in veterinary medicine relates to nontherapeutic use (metaphylaxis and growth promotion) and overuse in disease control [22]. It has been estimated that up to 50 % of human antibiotic use and up to 80 % of veterinary antibiotic use could be eliminated without serious consequence [197].

Animals are treated to a lesser extent with antibiotics than humans: a 6.3-fold higher use of antibiotics in mg/kg of body mass per year has been estimated in humans [177]. However, the conditions of antibacterial use in farm animals exert a

high pressure for selection of resistance [1, 31]. More than 80 % of antibiotics are administered to food animals via oral flock treatment, in which whole animal herds are under long-term exposure to low levels of broad-spectrum antibiotics [177]. Low concentrations of antibiotics have been discovered to cause radical-induced random mutagenesis, which in turn creates multidrug resistance to antibiotics beyond the one used for treatment [96].

Nontherapeutic antibiotics are typically administered orally. Because antibiotics are typically poorly adsorbed in the gut, the majority is excreted in urine and feces [152]. Antibiotic metabolites can also be antimicrobially potent or can be transformed back to the parent compound [5, 152]. Antibiotic residues in urine and feces spread in the environment through wastewater or use as fertilizers. Together with antibiotics disseminated in the environment by other means (flushing, land application of waste, aquaculture, and plant spraying), these residues may assist in maintaining or developing antibiotic-resistant microbial populations [6, 37, 68, 152]. In addition, waste from farms, homes, and hospitals contains microbiota carrying antibiotic resistance genes that can spread in the environment [6, 112].

It is generally accepted that antibiotic therapy and growth promotion select for and increase the prevalence of antibiotic resistance in animal-associated microbiota [2, 22, 37, 126, 199]. It is, however, unclear whether the pool of resistance genes generated by antimicrobial use in food animals influences the prevalence of therapeutic failures in humans [32, 200]. The resistance problem in humans has mainly risen from human use, and antibiotic use in food animals may actually reduce the risk of zoonotic transmission of animal pathogens to humans [24, 32, 75, 135]. However, there are examples of human commensal and pathogenic isolates that are resistant to antibiotics used only in veterinary science, as well as evidence of transfer of human (multiresistant) pathogens to animals and vice versa [1, 87, 169, 181].

Food is considered to be the most important vector for spread of resistance between humans and animals [195]. Evidence exists that ingestion of food contaminated by resistant bacteria selected in animals may lead to transfer of resistance determinants to bacteria in the human gut or cause an infection in which therapy is compromised [6, 32, 173]. In addition, antibiotic residues in food products, a by-product of antibiotic use [5], may allow the selection of antibiotic-resistant bacteria after the food is consumed [114].

3 Control of Antibiotic Use

Growth enhancement use of antibiotics in the 1950s and 1960s led to an increase in antibiotic resistance in *Salmonella* strains associated with calf disease [56]. Emergence of resistance led the UK to set up the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, which in its 1969 report recommended that antibiotics with therapeutic value should not be applied

as growth promoters [85]. This "Swann Report" was the first action to begin the much-needed rationalization of antimicrobial use.

The World Health Organization (WHO) acknowledged the high importance of antimicrobial resistance as a threat to human and animal health by declaring antimicrobial resistance the topic of World Health Day 2011. WHO has devised a strategy for containment of antimicrobial resistance [191] as well as guidelines for rational drug use in humans [193, 194] and surveillance of antimicrobial resistance [192]. WHO has also created a ranking of antibiotics that are critically important for human medicine, which is intended to help develop prudent antimicrobial use in agriculture and veterinary medicine [195].

Responsible veterinary and agricultural use of antibiotics has been considered by international organizations such as Codex Alimentarius, World Organization for Animal Health, and World Veterinary Association, which have published guidelines for the prudent use of antimicrobial products in food animals [30, 125, 201]. Guidelines for the responsible use of antimicrobials in human medicine have been provided in the United States by the Centers for Disease Control and Prevention and in the EU by the European Council [38, 45, 69, 70]. Countries such as Brazil, South Korea, Canada, Australia, and New Zealand have also implemented policies and programs to prevent the emergence of resistance through antibiotic stewardship (i.e., appropriate antibiotic use) [45, 74, 124, 127, 203].

The EU devised a 5 year plan consisting of 12 key actions against antimicrobial resistance [51]. The plan noted that EU recommendations for prudent use in veterinary medicine should be introduced. Also, existing resistance monitoring programs in the EU require harmonization regarding antimicrobials surveyed, definition of resistance, and epidemiological cutoff values—that is, the minimum inhibitory concentrations used for designating wild-type and resistant strains [157]. The EU gradually enforced a total ban on the use of growth promoters in food animals, taking full effect in 2006 [47].

EU legislation enforces the establishment and execution of national monitoring plans in EU countries, under which a set percentage of animal products should be monitored for (antibiotic) residues and other contaminants [42]. This promotes the prudent use of antimicrobials as well as food safety by ensuring residues do not reach consumers. An EU council regulation for the establishment of maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin became effective in 1990 and was repealed in 2009 by an update, which recognized progress in detection methods and pharmacological, toxicological, and microbiological effect assessments on establishing MRLs [48, 53]. In 2010, MRLs of pharmacologically active substances were combined under a single commission regulation [49].

The annual report on the execution of EU national monitoring plans in 2009 [50] reported that 445,968 samples were tested under the monitoring plans, fulfilling the requirements of the minimal amount of samples to be tested [42, 43]. Of these, 155,432 samples (34.9 %) were tested for presence of antibacterials, and 332 samples (0.21 %) were found to be noncompliant (i.e., containing a

concentration above the MRL). Of the noncompliant samples, 109 were found in pigs, 68 in bovines, 50 in milk, 32 in poultry, and the rest in sheep/goats (28), honey (23), rabbits (9), aquaculture (9), horses (2), farmed game (1), and eggs (1). Honey had the highest prevalence of noncompliance, with 23 (0.98 %) samples out of 2,336 testing positive.

4 Detection Methods of Antibiotic Residues in Food

Antibiotics are one of the most significant groups of food contaminants [92]. The occurrence of antibiotic residues is determined by the pharmacokinetic distribution and removal rate of the drug, extended use and dosage, route of administration, contamination of feed or water, and physical condition of the animal [5, 152]. To avoid the appearance of residues in food, withdrawal periods have been assigned to various antibiotics based on pharmacokinetic data [44, 48, 53]. The withdrawal period is the span of time until a safe level in edible tissues and other products (milk, eggs, honey) is achieved. EU MRLs are the points of reference for the establishment of withdrawal periods [44, 48, 53].

European Commission Decision 2002/657/EC lays down performance and validation criteria for the screening and confirmatory methods used in national residue monitoring programs [45]. Methods of analysis of antimicrobials can be grouped into three categories: microbiological, immunochemical, and physicochemical [50]. Microbiological methods are fast screening methods allowing a high sample throughput, but limited information is obtained on substance identity and concentration in the sample. Immunochemical methods are rapid, selective, and sensitive; they are widely applied in some areas of residue analysis, typically in screening for substances that cannot be discerned by microbiological growth inhibition. Physicochemical methods allow for accurate identification and quantification of the substance, and they are therefore applied in confirmatory analysis of suspect samples identified by screening methods.

4.1 Confirmatory Analysis of Antibiotic Residues

Physicochemical methods are typically used in confirmatory analysis of the presence and concentration of antibiotic residues in products of animal origin after they have been indicated by a screening test [50]. A confirmatory test involves a more sophisticated testing method providing full or complementary information that enables the substance to be identified precisely and confirms that the MRL has been exceeded [50]. Confirmatory methods are typically not suitable for screening because they are time-consuming, expensive, and require complex laboratory equipment as well as trained personnel [26]. Also, they typically require extensive

sample preparation based on liquid and solid-phase extraction and multi-step clean-up [94].

European Commission Decision 2002/657/EC lists suitable methods for quantitative confirmatory analysis of antibiotic residues [46]. These consist of chromatographic separation in combination with detection (Table 2). The decision also introduces identification points (IP), the idea of which is that a laboratory is allowed to use any technique or combination of techniques to earn a minimum number of IPs necessary for proper identification of a component [165]. As a consequence, methods based on chromatographic analysis followed by mass spectrometric detection are becoming the norm in identifying and quantifying antibiotic residues [19, 115, 166].

Alternative physicochemical methods for confirmatory analysis include capillary electrophoresis, which has been used to detect antibiotics in food matrices [62]. However, although the technique is less expensive and has higher separation efficiency than high-performance liquid chromatography (HPLC) methods, the lower sensitivity of capillary electrophoresis may prevent detection at MRL [83, 115]. Immunoanalytical methods such as radioimmunoassays, fluoroimmunoassays, and the most commonly used enzyme-linked immunosorbent assays (ELISA) are quantitative and have a high sensitivity, capacity for high-throughput, and often do not require complex sample clean-up [129]. However, immunoassays are generally group-specific by nature and therefore cannot offer direct identification of the analyte due to cross-reactivity towards structurally similar antibiotics [99]. Thus, they are often better suited for use as screening methods.

Liquid chromatography (LC)–mass spectrometry (MS) methods form the majority of routine confirmatory methods, but other methods are also validated along the 2002/657/EC guidelines. Table 3 presents a selection of validated confirmatory methods for two antibiotic classes, tetracyclines (TC) and macrolide/streptogramin/lincosamide (MLS) antibiotics, and briefly describes sample preparation and clean-up steps necessary for each method. Tetracyclines are the most commonly used class of veterinary antibiotics. In a study covering 10 European countries, tetracyclines accounted for 48 % of total sales of veterinary antibacterial agents in 2007 [73]. The WHO ranks antimicrobials in the three-class system (critically important, highly important, and important antimicrobials) based on their importance for human medicine [195]; tetracyclines are classified as a critically important antibiotic. Macrolides, lincosamides, and streptogramins (MLS) are placed in the same antibiotic class due to their similar modes of action and resistance patterns [148, 174, 179, 196]. Depending on the country, MLS is the third or fourth most used class of veterinary antimicrobials in Europe [55]. Macrolides are among the top three critically important antimicrobial groups, along with quinolones and third- and fourth-generation cephalosporins [195]. Streptogramins are listed as critically important and lincosamides as important to human medicine.

Most methods described in Table 3 represent multiresidue methods that can detect antibiotics from other classes than TC and MLS groups [19, 23, 29, 35, 71, 72, 108, 134, 167, 171], but results focus on only these two classes. The methods mainly

Table 2 Suitable confirmatory methods for veterinary drugs or contaminants in products of animal origin according to European Commission Decision 2002/657/EC [46]

LC or GC with mass-spectrometric detection	2-D TLC-full-scan UV/VIS
LC or GC with IR spectrometric detection	GC-electron capture detection
LC-full-scan DAD	LC-immunogram
LC-fluorescence	LC-UV/VIS (single wavelength)

LC liquid chromatography; *GC* gas chromatography; 2-*D* two-dimensional; *IR* Infrared spectrometry; *DAD* diode array detection; *TLC* thin layer chromatography; *UV/VIS* ultraviolet/visible spectrophotometry

originate from different EU countries and have been validated for various food matrices in order to be applied in carrying out the national residue monitoring plan.

European Commission Decision 2002/657/EC states that as a part of assay validation, a decision limit (CCα) must be established for confirmatory methods used for identification and quantification of substances with an established MRL. CCα is the limit at and above which a sample is considered to be noncompliant with an error probability α of 5 %. Therefore, it can be concluded with 95 % certainty that a sample is noncompliant. Also, detection capability (CCβ) must be established for confirmatory methods, although it is considered to be more important in validation of screening methods. Methods used for residue detection of substances with an established MRL, such as antibiotics, must have a CCβ with a false compliant rate <5 % [46]. In other words, CCβ is the smallest amount of analyte that can be detected in a sample with 95 % confidence. CCβ must be less than or equal to the MRL for less than 5 % of noncompliant samples to give a false compliant result.

To ease the comparison of various methods and their qualification for MRL standards, CCβ is given in Table 3 as a fraction of MRL. CCα corresponds to the limit of quantification and CCβ to the limit of detection, which were used in assay validation prior to Decision 2002/657/EC and were determined using various methods. The guidelines for establishing CCα and CCβ in Decision 2002/657/EC omit this variation.

4.2 Screening Methods for Antibiotic Residues

Screening is used for large sample numbers to pinpoint suspect noncompliant samples to be subjected to confirmatory analysis. Methods used for screening can detect an analyte (family) at the MRL level, providing semi-quantitative or qualitative results [26]. The main requirements for a screening method include rapidity, ease of use, low set-up and running costs, high-throughput capacity, repeatability, and high sensitivity (low amount of false negatives) and specificity (low amount of false positives) [175].

Methods used for screening for antibiotic residues include immunoanalytical methods and biosensors, as well as methods typically used for confirmatory

Table 3 An overview of confirmatory methods validated according to Commission Decision 2002/657/EC for macrolide/lincosamide/streptogramin (MLS) and/or tetracycline (TC) group antibiotics

Analytical method	Sample matrix	Sample preparation	Sample clean-up	Recovery (%)	CCβ (xMRL)	Analytes	Ref.
UHPLC-MS/MS	Chicken muscle	LE with 1 % acetic acid in ACN/H_2O (80:20 v/v); LLP by $MgSO_4$ and sodium citrate	Dispersive SPE with PSA sorbent; filtration	75–117	1.1–2	4 M	[108]
UHPLC-MS/MS	Milk	LE with ACN	Filtration	58–128 (MLS)	n.r.	11 M, 2 L, 1 S	[171]
HPLC-ESI-MS/MS	Animal feed	Ultrasonic LE CH_3OH/CH_3CN/McIlvaine buffer pH 4.6 (37.5:37.5:25 v/v/v)	Dispersive SPE with PSA sorbent	51–63 (TC) 88–109 (MLS)	n.r.	3 TC 3 M, 1 L, 1 S	[19]
LC-ESI-MS/MS	Fish tissue	Ultrasonic LE with ACN/MeOH (1:1 v/v) +0.05 % v/v formic acid	Filtration	40–100	0.1–0.3	5 TC	[35]
LC-MS/MS	Milk and bovine muscle	Pressurized LE with ASE 200; LE of lipids with ether	Filtration	70–93	1.1–1.6	5 M, 2 L	[91]
HPLC-DAD	Bovine muscle	LE with MeOH/succinic acid buffer (1:1 v/v)	Column MCAC; McIlvaine buffer pH 3 elution; cartridge SPE; MeOH elution	91–104	1.2–1.6	4 TC, 3 epi-TC	[33]
LC-MS/MS	Bovine and porcine muscle	LE with MeOH/H_2O (7:3 v/v)	–	61–70 (TC) 75–84 (M)	1.4–1.9 (TC) 1.3–1.4 (M)	4 TC 4 M	[72]
HRLC-TOF-MS	Muscle (various species), fish tissue (various species), eggs	LE with ACN/H_2O (6:4 v/v)	Column SPE; MeOH/ethyl acetate (1:1 v/v) elution for egg or MeOH/ACN (1:1 v/v) elution for muscle and tissue	63–120 (TC) 69–261 (ML)	≤2 (TC and ML)	4 TC 6 M, 2 L	[134]

(continued)

Table 3 (continued)

Analytical method	Sample matrix	Sample preparation	Sample clean-up	Recovery (%)	CCβ (xMRL)	Analytes	Ref.
LC-MS/MS	Bovine and porcine muscle	Pressurized LE with ASE 200	–	73–86 (TC) 82–91 (ML)	1.1–1.2 (TC) 1.1–1.2 (ML)	3 TC 4 M, 1 L	[23]
UHPLC-ESI-MS/MS	Chicken muscle	LE with MeOH/H_2O (7:3 v/v)	Filtration	87–97	1.1–1.2 (TC) 1.1–1.2 (M)	4 TC 4 M	[29]
UHPLC-TOF-MS	Milk	LE with ACN	Column SPE; MeOH elution	n.r.	1.3–1.4 (TC) 0.3–1.3 (ML)	4 TC 6 M, 2 L	[167]
LC-DAD	Liver and kidney (various species)	LE with McIlvaine buffer pH 3.5	Cartridge SPE; MeOH elution	40–88	1.0–1.3	7 M	[13]
HPLC-DAD	Milk	LE with TFA/oxalate buffer (1:10 v/v)	Cartridge SPE; elution with CH_3OH/CH_3CN/0.01 M oxalic acid (30:30:40 v/v/v)	98–111	1.0–1.1	7 TC	[150]
LC-MS/MS	Muscle and kidney (various species)	LE with MeOH/H_2O (7:3 v/v)	–	26–62 (TC) 44–104 (M)	Below MRL	4 TC 4 M	[71]
HPLC-DAD	Bovine muscle	LE with citrate buffer pH 4	Cartridge SPE; elution with CH_3OH/CH_3CN/0.05 M oxalic acid (30:30:40 v/v/v)	91–104	1.1–1.2	5 TC	[149]

ACN acetonitrile; *ASE* 200 accelerated solvent extraction system; *DAD* diode array detection; *epi-TC* 4-epimer metabolite of a tetracycline; *ESI* electrospray ionization; *HPLC* high-performance liquid chromatography; *HRLC* high-resolution liquid chromatography; *LC* liquid chromatography; *LE* liquid extraction; *LLP* liquid–liquid partitioning; *MCAC* metal chelate affinity chromatography; *MS* mass spectrometry; *n.r.* not reported; *PSA sorbent* primary-secondary amine sorbent; *SPE* solid-phase extraction; *TFA* trifluoroacetic acid; *TOF* time-of-flight; *UHPLC* ultra-high-performance liquid chromatography

analysis (LC-MS, LC-ultraviolet/visible spectrophotometry, LC-fluorescence, LC-diode array detection) [26, 134, 158, 168, 175, 184]. Most screening tests are, however, based on microbial growth inhibition. In 2001–2003, 15 EU reference laboratories reported that 53 % of muscle sample screening was performed using microbiological methods, and the second most common method, ELISA, was used in 21 % of cases [184]. A disadvantage of microbiological assays is that they cannot establish the identity of a compound, although they can be fairly group-specific [136, 138]. However, they are cost-effective in situations where most samples are expected to be compliant [137]. Microbial growth inhibition assays are also suited for high-throughput, require no high-tech equipment or specialized technicians, and (due to their general nature) may detect unknown or new compounds lacking from the confirmatory method toolkit [20].

Growth-inhibition assays mainly come in two formats: the tube test and the (multi-)plate assay. In the tube test, the growth of indicator bacteria in the test medium causes a pH-indicator color change, which is absent during growth-inhibition [65, 106]. Simple use and commercial availability of tube tests has caused them to be widely applied both in the laboratory and in the field [20]. A plate test uses an agar plate inoculated with the test organism [60, 67]. Diffusion of analyte into the agar causes a growth inhibition zone whose diameter depends on analyte concentration. Detection of all veterinary-relevant antibiotics requires multiplate assays with conditions suitable on each plate for detection of one or select groups of antibiotics [139].

According to European Commission Decision 2002/657/EC, a screening method must have a CCβ with a false compliant rate of <5 % [46]. A guideline document by EU Reference Laboratories details screening method validation through determination of stability, applicability, and ruggedness, as well as selectivity and specificity [9]. Stability of the analyte and standard samples must be determined under various storage conditions. Applicability refers to usability in various sample matrixes, and ruggedness is the method's ability to withstand minor variations occurring during laboratory analysis, such as the age of reagents, temperature fluctuations, and personnel changes. Selectivity and specificity refer to the power of discrimination between the analyte and coexisting (related) substances. Table 4 presents microbial screening methods validated along these guidelines.

Establishment of MRLs and performance criteria of analytical methods [46, 49, 53] was followed by a critical evaluation of the commonly used screening methods [67, 136, 138, 141]. As Decision 2002/657/EC allows screening method development following the validation criteria, national monitoring plans are based on a variety of screening methods. The EU Four-Plate Test (EU4pt) [17] was considered a gold standard for a long time, but it has now been deemed insufficiently sensitive (although it is still widely in use) [12]. Two commonly used commercial tube tests, Premi® Test and Delvotest® SP-NT, lack sufficient detection capabilities for several antibiotic groups, including tetracyclines [65, 106]. In addition, the Premi® Test suffers from a high false-positive rate [141].

Validation of the Screening Test for Antibiotic Residues (STAR) used by the French national residue monitoring plan showed that the CCβ values of most

Table 4 Microbial growth-inhibition screening methods validated according to CD 2002/657/EC

Test	Test format	Indicator organism	Analytes	Selective conditions and supplements	Sample matrixes	CCβ (xMRL)	Ref.
Two-plate microbiological method	Multi-plate	*Bacillus subtilis* BGA	TC,Q	pH 6, NaOH	Shrimp	0.28–0.65 (4TC)	[34]
		B. subtilis BGA	S	pH 7.5, TMP, PABA			
Screening Test for Antibiotic Residues (STAR)	Multi-plate	*B. subtilis* BGA	TC, AF	pH 8	Milk[b], muscle	1–2.5 (2TC)	[67]
		Kocuria rhizopila ATCC 9,341	M, C	pH 8		2–5 (2 M, 1 L)	
		Bacillus cereus ATCC 11,778	TC	pH 6			
		Escherichia coli ATCC 11,303	Q, C	pH 8			
		Bacillus stearothermophilus ATCC 10,149	B, S, C, Q, ML, AG, AF				
Explorer®	Microtiter plate	*Geobacillus stearothermophilus*	S, TC, M, AG	–	Eggs	7.5–20 (4TC) 2–2.5 (2 M)	[66]
Premi®Test	Tube	*B. stearothermophilus*	S, TC, M, AG	–	Eggs	1–10 (4TC) 0.25–0.5 (2 M)	[66]
Nouws Antibiotic Test (NAT) Post-screening[a]	Multi-plate	*B. cereus* ATCC 1,178	TC	pH 6, 30 °C	Muscle, kidney	0.25–0.5 (4TC) 0.07–1 (5 M, 2 L)	[138, 141]
		K. rhizopila ATCC 9,341	B, ML	pH 6, TY, CX, 30 °C			
		Yersinia ruckeri NCIM 13,282	Q	pH 6.5, 30 °C			
		Bacillus pumilus CN 607	AG	pH 8, 37 °C			

(continued)

Table 4 (continued)

Test	Test format	Indicator organism	Analytes	Selective conditions and supplements	Sample matrixes	CCβ (xMRL)	Ref.
Nouws Antibiotic Test (NAT)	Multi-plate	*B. cereus* ATCC 1,178	TC	pH 6, CAP, 30 °C	Renal pelvis fluid	0.013–0.08 (4TC)	[136]
Initial screening[a]		*K. rhizopila* ATCC 9,341	B, ML	pH 8, 37 °C		0.5–4 (5 M, 2 L)	
		Yersinia ruckeri NCIM 13,282	Q	pH 6.5, 30 °C			
		Bacillus pumilus CN 607	S,D	pH 7, TMP, 37 °C			
		B. subtilis BGA	AG	pH 8, 37 °C			
Delvotest® SP-NT	Tube	*B. stearothermophilus*	B, S, C, AG, TC	–	Milk	2 (1TC)	[106]

Detection capability CCβ is reported as a fraction of the MRL for only tetracycline (TC) and macrolide/lincosamide (ML) antibiotics

AG aminoglycosides; *AF* amphenicols; *B* β-lactams; *C* cephalosporines; *CAP* chloramphenicol; *CX* cloxacillin; *D* diaminopyridines; *M* macrolides; *ML* macrolides and lincosamides; *n.r.* not reported; *PABA* 4-aminobenzoic acid; *Q* quinolones; *S* sulfonamides; *TC* tetracyclines; *TMP* trimethoprim; *TY* tylosin

[a] *NAT* consists of an initial screening performed to renal pelvis fluid samples, and a corroborating post-screening step for suspect samples using one of the four plates described and muscle or kidney fluid as sample

[b] *STAR* has been validated for milk [64] following an intralaboratory procedure and not Decision 2002/657/EC criteria. Validation for muscle was done following 2002/657/EC

antibiotics tested, including tetracyclines and macrolides/lincosamides, were above the EU MRL values [64, 67]. In addition, group specificity was not achieved. Improved group-specificity was attained in the six-plate combined plate microbial assay (CPMA), but a false compliant rate of <5 % was only partially achieved [60]. The Nouws antibiotic test (NAT) used by the Dutch national residue monitoring plan yields group-specific detection and shows below- or near-MRL sensitivity towards most veterinary antibiotics [136, 138]. However, because initial screening is performed to renal pelvis fluid extracted from the kidney, high residue levels occurring solely in muscle may never make it to the postscreening step [141]. NAT has a higher workload due to the extra postscreening step, which the CPMA test has been designed to avoid [60]. On the other hand, post-screening reduces the number of samples subjected to costly confirmatory analysis [138, 139].

The Finnish national monitoring plan uses microbial growth inhibition for the majority of screening. Out of 6,900 samples tested for the presence of antimicrobials in 2005, 83.6 % were processed with microbial growth inhibition and 16.4 % with physicochemical methods [151]. For porcine and bovine kidney or muscle samples, a two-plate test was used with *B. subtilis* BGA as the indicator organism and Delvotest®SP-NT for milk samples. In the view of studies discussed here, these methods are not likely to detect all noncompliant samples. The widespread use of insufficiently sensitive methods is reflected by a proficiency test involving 23 laboratories performing residue screening in the EU [12]. The false-negative rate for microbial methods was 73 % compared to 22 % for chemical methods, and only 39 % of the laboratories identified the test samples correctly.

5 Biosensors for Detection of Antibiotics in Food

Biosensors are an emerging class of methods suitable for screening purposes. By definition, biosensors combine a biological recognition element with a transducer to produce a measurable signal proportional to the concentration of the analyte [117, 183]. Figure 1 presents a general working principle of biosensors and the types of recognition elements and transducers typically used in biosensors.

Although biosensors are mostly used for antibiotic detection in the environment, they are also increasingly used in screening for antibiotic residues in food, currently in 8 % of screening cases [26, 144]. Table 5 gives an overview of biosensors developed for antibiotic detection in food. Biosensor assays have a high capacity for automatization and high throughput, produce results rapidly, and typically require no or very simple sample pretreatment [86, 175]. Limitations of biosensor methods include instability of the biorecognition element due to the conditions (pH, ionic strength, temperature) it is exposed to during immobilization and the assay [26]. Even so, biosensor methods often are robust enough to allow regeneration, so successive cycles of analysis can be performed with the same recognition molecules [4, 21, 58, 110, 116]. Surface plasmon resonance (SPR) appears to be the transducer of choice for antibiotic detection, as it is utilized in

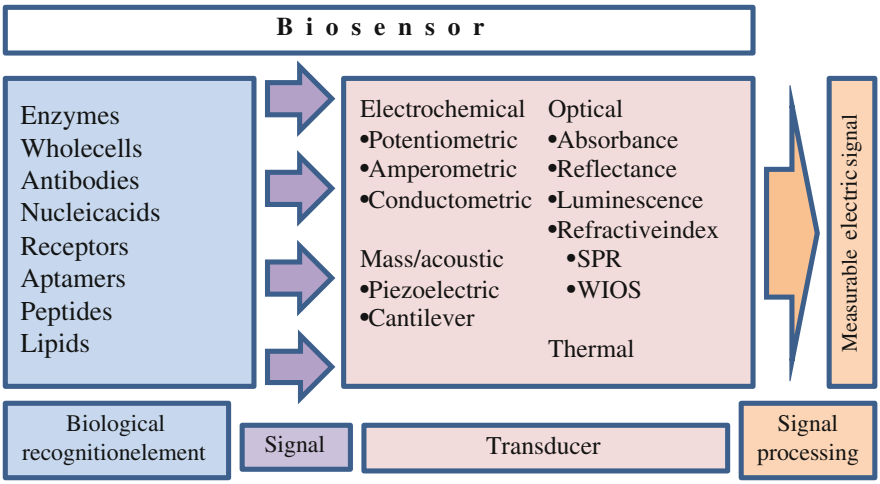

Fig. 1 The principle of biosensors. Biosensors combine a biomolecule-based recognition element with a transducer, which converts the signal triggered by the recognition event to a quantifiable electric signal [117, 183]. *SPR* surface plasmon resonance; *WIOS* wavelength-interrogated optical system

49 % of published detection methods [144]. SPR allows easy-to-use, real-time, label-free studying of biomolecular interactions [86].

In accordance with the role of biosensors as an emerging screening tool, a few biosensor screening methods have been validated according to 2002/657/EC [10, 21, 161]. Biosensor methods are typically suitable only for screening due to cross-reactivity within antibiotic groups. However, a biosensor using an ssDNA aptamer as the recognition element has been reported to specifically detect tetracycline among tetracycline antibiotic family members [93]. An interlaboratory study compared a SPR biosensor screening assay for fluoroquinolones in various food matrices with established microbiological growth inhibition and LC-MS/MS methods [190]. The study demonstrated that unlike the microbiological assay, the biosensor method correctly identified all samples and demonstrated advantages in sensitivity and analysis time. However, assay costs were higher using the biosensor assay (30–50 €/sample) than the microbiological method (5–15 €/sample), which may curb the interest in SPR-based biosensor screening methods.

To establish biosensors as a screening method for antibiotic residues, they have been studied in combination with confirmatory methods for simultaneous development of a comprehensive detection procedure. Ashwin et al. [10] developed an SPR biosensor screening and LC-MS/MS confirmatory method for chloramphenicol residues in four different food matrices and performed validation of the method according to 2002/657/EC. Marchesini et al. [110] developed a dual SPR biosensor assay, where suspect samples from the first round of SPR were subjected to HPLC fractionation, a second round of SPR, and finally LC–electrospray

Table 5 An overview of biosensors for antibiotic detection

Biological recognition element	Recognition event	Transducer	Sample matrix	Sample pretreatment	Analyte	Sensitivity (LOD)	Ref.
DD-carboxypeptidase	Inhibition	SPR	Milk	Dilution	7 B	0.5–2 × MRL	[76]
β-lactamase	Hydrolysis	Amperometric pH detector	Milk	Protein removal by salting-out	1 B	2,000 × MRL	[28]
Polyclonal antibody	Binding	SPR	Honey	LLP with potassium buffer/hexane (5:3 v/v), SPE, MeOH elution	TY	2.5 μg/kg[a]	[21]
Polyclonal antibody	Binding	SPR	Chicken muscle	Homogenization, ultrafiltration, clean-up with SPE, MeOH/ACN (8:2) elution	6 FQ	<MRL	[110]
Antibody	Binding	SPR	Milk, honey, prawn, porcine kidney	Varies depending on the matrix, includes LE, LLP, SPE	CAP	0.3–0.7 × MRPL[b]	[10]
Monoclonal antibody	Binding	SPR	Milk	Hydrolysis of the β-lactam ring	9 B	≤MRL	[63]
Monoclonal antibody	Binding	SPR	Chicken serum	Dilution	8 S	0.07–0.2 × MRL	[77]
Monoclonal antibody	Binding	Bioluminescence	Milk	–	2 B, 1 C, 2 S, 3 AG, 2 M	0.002–0.8 × MRL	[95]
Monoclonal antibody	Binding	SPR	Milk	Dilution and filtration	1 FQ, 1 S, CAP	0.02 × MRL 3.6 × MRPL[b]	[58]
Antibody and receptor	Binding	WIOS	Milk	Dilution	1 S,1 FQ, 1 B, 1 TC	0.005–0.8 × MRL	[4]

(continued)

Table 5 (continued)

Biological recognition element	Recognition event	Transducer	Sample matrix	Sample pretreatment	Analyte	Sensitivity (LOD)	Ref.
Repressor protein	Binding	SPR	Milk, honey	Dilution (honey); heating and centrifugation (milk)	7 TC, 3 epi-TC	0.15–0.25 × MRL	[119]
SBP (sulfonamide binding protein)	Binding	SPR	Porcine muscle	Homogenization, liquid extraction	20 S	0.2 × MRL	[116]
ssDNA aptamer	Binding	Voltammetric	Buffer	–	1 TC	4 μg/kg[c]	[93]
Microbial cell	Growth inhibition	Potentiometric CO_2 detector	Milk	–	7 Q, 3 TC	0.25–0.8 × MRL	[130]

The references were chosen on the basis of demonstrated applicability in a food matrix, except Kim et al. [93], who introduced a new interesting recognition element, a ssDNA aptamer, in antibiotic biosensors

ACN acetonitrile; *AG* aminoglycosides; *B* β-lactams; *C* cephalosporines; *CAP* chloramphenicol; *epi-TC* 4-epimer metabolite of a TC, *FQ* fluoroquinolones; *LE* liquid extraction; *LLP* liquid-liquid partitioning; *LOD* limit of detection, *M* macrolides; *MeOH* methanol; *Q* quinolones; *S* sulfonamides; *SPE* solid-phase extraction; *SPR* surface plasmon resonance; *TC* tetracyclines; *TY* tylosin; *WIOS* wavelength-interrogated optical system

[a] No EU MRL for tylosin in honey has been set [49] so no detectable residues are allowed

[b] The use of chloramphenicol in treatment of food animals in the EU is prohibited. Therefore, an MRL is not set. The EU minimum required performance limit (MRPL) for chloramphenicol residues in food products of animal origin is 0.3 μg/kg [46, 47]

[c] Not established in a food matrix and therefore cannot be compared to an MRL

ionization–time of flight–MS confirmatory analysis to identify and quantify residues in positive fractions harboring fluoroquinolone receptor binding activity.

Future directions in antibiotic biosensor development include assay multiplexing and portable devices for field use [86]. As an example of multiplexing, recently developed SPR biosensor microarrays simultaneously detect on a single sensor chip two aminoglycoside antibiotics or compounds from four major antibiotic families: aminoglycosides, sulfonamides, amphenicols, and fluoroquinolones [142, 143]. A biosensor based on a wavelength-interrogated optical system transducer can simultaneously detect sulfonamide, fluoroquinolone, β-lactam, and tetracycline antibiotics on a multianalyte sensor chip [4]. Portable multiplex SPR biosensors have been developed for onsite analysis of milk samples for fluoroquinolone family compounds or sulfonamide, chloramphenicol, and fluoroquinolone residues [58, 59]. Commercialization of biosensors requires wireless technology, automatization, and miniaturization, which also must be future directions of antibiotic biosensor development [109].

Proteins (i.e. enzymes and bioreceptors) have traditionally been used as biological recognition elements in antibiotic biosensors. A new type of recognition element, a DNA-based aptamer, was recently introduced in tetracycline detection [93]. Proteins can also be modified for improved biosensor performance: a fluorescein-labeled β-galactosidase mutant with reduced catalytic activity was used as a recognition element for β-lactams in a fluorescence-based biosensor [27].

5.1 Whole-Cell Biosensors

Whole-cell biosensor assays are an emerging bioactivity-based screening method for antibiotic residues [20]. The principle is more widely applied in environmental monitoring [36, 82, 105, 122, 198] but the amount of food control applications is increasing [41, 78, 90, 102–104, 186]. In whole-cell biosensors, the living cell functions as the biological recognition element, which in the event of biosensing produces a specific signal to be transduced into a quantifiable electrical signal [36, 41].

Whole-cell biosensor bacteria can be divided in systems with constitutive or inducible expression [36, 198]. The former has a high continuous expression of signal, which decreases under toxic conditions (“turn off”). This type of detection is highly nonspecific, as signal decrease is a result of any type of cytotoxic effect [7, 185]. Inducible expression, however, is more specific, as transcription of the reporter gene occurs only when the stimulus is present (Fig. 2). Specificity is achieved by employing a promoter-regulatory protein pair that recognizes and reacts to the stimulus (“turn on”) [36, 170].

Inducible whole-cell biosensors can be further divided into effect- and compound-specific sensors [36, 198, 202]. The former are stimulated by a change in a physicochemical condition (pH, temperature, osmotic pressure, electron potential) or specific type of toxicity (DNA, protein or membrane damage, or oxidative

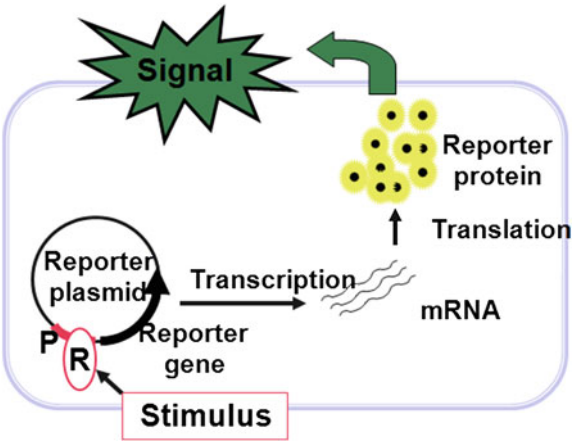

Fig. 2 Operating principle of an inducible bacterial whole-cell biosensor. The stimulus induces reporter gene expression, which leads to quantifiable signal. Regulatory protein R limits induction to occur from promoter P only when the stimulus is received. Induction involves relieving repression or activating transcription

stress) by coupling the reporter gene to a promoter involved in the stress response [155, 182]. Compound-specific sensors react to a single compound or group of compounds with similar chemical characteristics or mode of action [100, 187]. Response of the sensor strains correlates with the concentration and potency of inducing compounds [78, 121].

Whole-cell biosensor assays offer a possibility for more cost-effective and accurate group-specific detection than microbial growth-inhibition methods and are better suited for high-throughput due to assay miniaturization from agar plates to microtiter plates [26, 140]. Also, growth inhibition on agar plates is typically visualized after overnight incubation, whereas whole-cell biosensor assays can be performed within hours [20, 198]. Whole-cell biosensors can equal growth inhibition assays in below-MRL sensitivity and simplicity in sample preparation [140].

Biosensor assay ruggedness is advanced by cell preservation methods such as lyophilization, vacuum drying, and immobilization in biocompatible polymers [14]. These methods facilitate reagent-like use of the biosensor cells [102–104]. The fact that whole-cell biosensors inherently produce the necessary assay components, and just need the presence of the analyte (and sometimes substrate) to induce signal production, further enhances assay ruggedness [170]. The renewable storage of assay components within the biosensor cell helps to overcome the instability problems encountered with using purified biomolecules, such as enzymes, as recognition elements in biosensors [202].

There are some intrinsic disadvantages to using whole-cell biosensors. When purified biomolecules are used for recognition, conditions can be optimized for the biosensing event [4, 107, 132, 133, 189]. In contrast, biosensor cells continuously sense their local environment, and bioassay variation is caused by responses to diverse intra- and extracellular factors such as cell concentration, growth stage and metabolic activity, nutrient availability, temperature, pH, oxygen content, inducer type and bioavailability, and duration of induction [78, 111, 145, 155, 187]. However, with standardization of assay conditions and applying homogeneous

biosensor cell material through lyophilization, reproducible results can be achieved [102–104, 159].

Whole-cell biosensors typically have a narrower detection range than biomolecule-based antibiotic assays because the toxicity of the analyte to the cell at high concentrations causes a characteristic hook effect seen as a bell-shaped dose-response curve [61]. Assay conditions must therefore be optimized so that the dynamic range of the assay meets the MRL [103, 104].

Because in biosensor cells the biorecognition elements typically reside within the cell, the analyte must first pass the diffusional barrier cell wall—a rate-limiting step in the biosensing reaction that leads to lowered sensitivity [182]. Using permeabilizing agents or host strains with a defective outer membrane permeability barrier can facilitate more efficient analyte entry into cell [101, 121, 186]. Bacterial cells also have group-specific and multidrug mechanisms of antimicrobial resistance, which may hinder intracellular accumulation of the antibiotic analyte [146–148, 172]. Choosing or creating host strains deficient in antimicrobial resistance mechanisms alleviates this problem [154].

The choice of reporter gene is yet another factor affecting whole-cell biosensor performance. The most commonly used reporters—luciferase (bacterial or eukaryotic), green fluorescent protein (GFP), and the enzyme β-galactosidase—all have their advantages and disadvantages when compared to each other (Table 6). Light can be measured from bacterial cells noninvasively and sensitively. Also, because of their high sensitivity and fast response times, luciferase reporters have found use in numerous biosensors, especially online monitoring systems [122, 182, 198]. GFP and β-galactosidase both suffer from a high cellular background. However, they benefit from higher stability compared to luciferases and require no ATP for signal production [105, 202].

5.2 *Inducible Whole-Cell Biosensors for Antibiotic Detection*

Inducible whole-cell biosensors for antibiotics include effect-specific and compound-specific sensors (Table 7). Effect-specific biosensors are induced by a stress reaction caused by the mechanism of action of different antibiotic classes. As an example, a panel of *E. coli*–based biosensors includes strains induced by cold shock response to translation inhibition (*cspA* promoter; amphenicol and tetracycline antibiotics), heat shock response to translation inhibition (*ibp*; aminoglycosides), SOS response to DNA replication inhibition (*sulA*; quinolones), and heat shock response to membrane damage and peptidoglycan synthesis interference (*P3rpoH*; β-lactams, polymyxins) [15, 154, 155]. A similar system based on *B. subtilis* whole-cell biosensors responds to antibiotic interference of the five major biosynthetic pathways of bacteria: biosynthesis of DNA, RNA, proteins, cell wall, and fatty acids [88, 178]. The antibiotic-inducible promoters were found by

Table 6 A comparison of reporter genes and proteins commonly used in whole-cell biosensors

Gene	Protein	Origin	Advantages	Disadvantages
lux	Bacterial luciferase	Luminescent bacteria	Rapid response High sensitivity No exogeneous substrate requirement	Heat lability (not in *Photorhabdus luminescens lux*-genes) Oxygen and ATP requirement
luc	Insect luciferase	Firefly, click beetle	Rapid response Very high sensitivity Heat resistance	Exogeneous substrate requirement Oxygen and ATP requirement
gfp	Green fluorescent protein	*Aequorea victoria* (jellyfish)	No substrate or ATP requirement Limited oxygen requirement High stability	Low sensitivity Lag time before expression Slow maturation Autofluorescence background
lacZ	β-galactosidase	*E. coli*	Detection by naked eye Good stability No ATP requirement	Exogenous substrate requirement Modest sensitivity Endogenous background

Adapted from [36, 105, 182, 202]

Table 7 An overview of effect- and compound-specific inducible whole-cell biosensors for antibiotics

Type	Analyte	Effect/Regulatory protein	Promoter	Reporter gene(s)	Host	Limit of detection[a]	Induction time	Remarks	Ref.
Effect-specific	Fluoro-quinolones	DNA replication interference	*pL*	*luxCDABE*	*E. coli*	n.d.	60–90 min	Heat induces *pL* → reporter plasmid replication, whose inhibition decreases signal	[8]
	DNA-damaging agents	SOS response	*cda* *recA* *sulA* *umuDC*	*gfp*	*E. coli*	n.d.	90–120 min		[123]
	Different antibiotic classes	Translation inhibition DNA replication interference	*cspA* *sulA*	*lucFF* *lucR1*	*E. coli*	1,000 CAP 50 OFL	3 h	Dual reporter strain *cspA*::*lacZ sulA*::*lucR1* detected both effects	[155]
	Vast majority of antibiotic classes	DNA biosynthesis RNA biosynthesis Protein biosynthesis Cell wall biosynth. Fatty acid biosynth.	*yorB* *yvgS* *yheI* *ypuA* *fabHB*	*lucFF*	*B. subtilis*	n.d.	3 h 90 min 4 h 1 h 3 h	The panel targets the majority of antibiotic classes. However, aminoglycosides, for example, did not induce *yheI*	[178]
	Different antibiotic classes	DNA damage Heat-shock Quorum sensing	*recA* *grpE* *lasI*	*luxCDABE*	*E. coli*	50–5 × 10^5	n.r.	Response signature of growth and/or luminescence inhibition and induction indicates mode of action	[54]

(continued)

Table 7 (continued)

Type	Analyte	Effect/Regulatory protein	Promoter	Reporter gene(s)	Host	Limit of detection[a]	Induction time	Remarks	Ref.
	β-lactams, glycopeptides D-cycloserine, bacitracin	Cell wall stress	*pbp2* *tcaA* *vraSR* *sgtB* *lytR*	*lacZ*	*S. aureus*	n.d.	2 h		[162]
	DNA-damaging agents	SOS response	*sulA*	*phoA*	*E. coli*	50 MMC 1700 NA	2 h	Sensitivity-enhancing host knockout mutations: enhanced cell membrane permeability (*rfaE*), inhibited DNA damage repair (*umuD*, *uvrA*)	[16]
Compound-specific	Tetracyclines	TetR	*tetA*	*luxCDABE*	*E. coli*	6	90 min		[100]
	Tetracyclines	TetR	*tetA*	*lacZYA* *luxCDABE* *gfp*	*E. coli*	10 10 < 20	3 h 50 min 16 h		[79]
	Tetracyclines	TetR	*tetA*	*gfp*	*E. coli*	50	16 h	FACS-optimized GFP mutant	[80]
	Tetracyclines	TetR	*tetA*	*gfp*	*E. coli*	5–16	18 h	Extended range biosensor created by insertion of *tet*(M) resistance gene	[11]
	Tetracyclines	TetR	*tetA*	*lacZ* *nuoA* *selA*	*E. coli*	110 2.6 1,450	2 h	Amperometric detection of cell respiration which is affected by expression of reporter genes	[160]

(continued)

Table 7 (continued)

Type	Analyte	Effect/Regulatory protein	Promoter	Reporter gene(s)	Host	Limit of detection[a]	Induction time	Remarks	Ref.
	Macrolides	MphR(A)	*mphA*	*luxCDABE*	*E. coli*	0.008	2 h		[121]
	Vancomycin	VanRS	*vanH*	*lacZ*	*B. subtilis*	100–1,000	4 h	Also responsive to other glyco-peptides, β-lactams, D-cyclo-serine, bacitracin, fosfomycin	[176]
	β-lactams	AmpR	*ampC*	*luxCDABE*	*E. coli*	2.5–2,500	3 h		[180]

CAP chloramphenicol; *gfp* green fluorescent protein gene; *FACS* fluorescence-activated cell sorting; *lacZYA lac* operon encoding β-galactosidase (*lacZ*), β-galactoside permease (*lacY*) and β-galactoside transacetylase (*lacA*); *lucFF Photinus pyralis* firefly luciferase gene; *lucR1* a red-shifted variant of firefly luciferase; *luxCDABE Photorhabdus luminescens* or *Vibrio fischeri* bacterial luciferase operon encoding luciferase (*luxAB*) and fatty acid reductase complex (*luxCDE*); *MMC* mitomycin C; *n.d.* not determined, *NA* nalidixic acid; *nuoA* NADH dehydrogenase I subunit A gene; *OFL* ofloxacin; *phoA* alkaline phosphatase gene; *selA* selenocysteine synthase gene

[a] In ng/ml. For effect-specific biosensors, the limit of detection is not relevant because multiple classes of antibiotics cause induction with various potencies

analyzing upregulated genes in an expression profile database of *B. subtilis* 168 [88, 89]; therefore, the regulatory proteins and pathways are not known.

Effect-specific biosensors can be used in seeking entirely new antibiotic mechanisms of action: bacterial cell division inhibiting compounds were discovered with a *B. subtilis* biosensor featuring two reporter genes to facilitate differentiation of specific and nonspecific inhibitors [163]. A downside of effect-specific biosensors regarding antibiotic residue detection is that they can detect analytes other than antibiotics that induce the same effect. For example, DNA-damaging agent-detecting SOS response biosensors are also induced by substances such as formaldehyde and hydrogen peroxide [16, 123].

Compound-specific biosensors for antibiotics offer more specific identification (Table 7). These whole-cell biosensors typically detect analytes in a group-specific manner; that is, they are responsive to a group of structurally similar antibiotics instead of a single compound. This kind of behavior is an advantage in screening because all or several compounds of an antibiotic family can be detected simultaneously [182]. Several TetR-based tetracycline-specific whole-cell biosensors have been constructed (Table 7). Some of these have also been applied for TC detection in a food matrix. Hansen and Sørensen [79] demonstrated applicability of a β-galactosidase-expressing biosensor in TC detection in incurred milk samples, whereas the bioluminescent biosensor by Korpela et al. [100] has been applied in milk [103, 104], porcine serum [102], fish tissue [131], and poultry tissue samples [140, 186]. All bioluminescence-based assays demonstrated below-MRL sensitivity towards TC residues in the various sample matrices used.

Quantitative determination of TC residues in fish tissue by a whole-cell biosensor assay has been demonstrated by good correlation with HPLC analysis [131]. However, Pikkemaat et al. [140] used the TC biosensor assay developed by Virolainen et al. [186] in routine screening analysis of poultry muscle samples and concluded that the assay only gave qualitative results. This was due to absorption of the bioluminescence signal by hemoglobin. The interference could be overcome by mathematical methods to account for the lost signal or by utilizing mutant versions of reporter proteins [84, 156], with emission maxima not overlapping with the hemoglobin absorption spectrum. The whole-cell biosensor assay [140] correctly identified noncompliant samples, but it indicated more suspect samples than the microbial inhibition test. This could be avoided by adjusting the cutoff value selected for differentiating between suspect and compliant samples. The whole-cell biosensor assay was faster, more sensitive, and more cost-effective than a routinely used microbial growth-inhibition assay. Market price per sample was € 15 with the microbial and €7.5 with the biosensor assay.

Because inducible whole-cell biosensor response to analytes is typically group-specific and depends on potency of the inducer, it is generally not possible to identify the analyte. However, Smolander et al. [159] developed an algorithm that allows direct identification of β-lactams inducing bioluminescence in a compound-specific biosensor. By following response trajectories over 300 min, it was possible to differentiate between 15 β-lactams. The classification system is scalable to larger sets of antibiotics of the same class or antibiotics of other classes than

β-lactams. This kind of an approach adds to the potential of whole-cell biosensors as a specific screening method for antibiotics of the same family. Accordingly, a study by Melamed et al. [113] combined a panel of antibiotic-inducible effect-specific whole-cell biosensors and an algorithm-based approach to compute patterns of response by various antibiotics to derive the identity of the inducing antibiotic.

6 Conclusions

The increase in microbial resistance to antibiotics seen during the last seven decades calls for stricter control of antibiotic use. Measures taken include guidelines for antibiotic stewardship in both human and veterinary medicine. To prevent occurrence of antibiotic residues in food, EU legislation enforces the establishment and execution of national monitoring plans, under which a set percentage of animal products should be monitored for (antibiotic) residues and other contaminants [42]. European Commission Decision 2002/657/EC lays down performance and validation criteria for the screening and confirmatory methods used in national residue monitoring programs [46]. Analysis methods for antimicrobials can be grouped in three categories: microbiological, immunochemical, or physicochemical [50]. Numerous methods belonging to each category have been validated according to the guidelines.

Screening for antibiotic residues in food is predominantly performed with microbial growth-inhibition assays. Biosensors are an emerging screening method, with some assays validated according to Decision 2002/657/EC. Currently, biosensor assays used for antibiotic detection are mostly based on SPR. However, whole-cell biosensors show promise as a robust, cost-effective, sensitive, and specific screening method. Using algorithm-based methods, specificity could be improved to the level where individual members of antibiotic families are directly identified. In the future, validation for use in routine analysis of food samples should be performed according to Decision 2002/657/EC [46]. In this way, whole-cell biosensors could gain a foothold among the screening methods available for antibiotic residue analysis, perhaps replacing the less specific and sensitive, more laborious, and voluminous microbial growth inhibition assays.

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Part IV
Applications of Bioluminescence in Health

Rapid In-vitro Testing for Chemotherapy Sensitivity in Leukaemia Patients

Elizabeth Anderson and Vyv Salisbury

Abstract Bioluminescent bacterial biosensors can be used in a rapid in vitro assay to predict sensitivity to commonly used chemotherapy drugs in acute myeloid leukemia (AML). The nucleoside analog cytarabine (ara-C) is the key agent for treating AML; however, up to 30 % of patients fail to respond to treatment. Screening of patient blood samples to determine drug response before commencement of treatment is needed. To achieve this aim, a self-bioluminescent reporter strain of *Escherichia coli* has been constructed and evaluated for use as an ara-C biosensor and an in vitro assay has been designed to predict ara-C response in clinical samples. Transposition mutagenesis was used to create a cytidine deaminase (cdd)-deficient mutant of *E. coli* MG1655 that responded to ara-C. The strain was transformed with the *lux*CDABE operon and used as a whole-cell biosensor for development an 8-h assay to determine ara-C uptake and phosphorylation by leukemic cells. Intracellular concentrations of 0.025 μmol/L phosphorylated ara-C were detected by significantly increased light output ($P < 0.05$) from the bacterial biosensor. Results using AML cell lines with known response to ara-C showed close correlation between the 8-h assay and a 3-day cytotoxicity test for ara-C cell killing. In retrospective tests with 24 clinical samples of bone marrow or peripheral blood, the biosensor-based assay predicted leukemic cell response to ara-C within 8 h. The biosensor-based assay may offer a predictor for evaluating the sensitivity of leukemic cells to ara-C before patients undergo chemotherapy and allow customized treatment of drug-sensitive patients with reduced ara-C dose levels. The 8-h assay monitors intracellular ara-CTP (cytosine arabinoside triphosphate) levels and, if fully validated, may be suitable for use in clinical settings.

Keywords Bioluminescent · Biosensor · Cytarabine · Leukaemia chemotherapy

E. Anderson · V. Salisbury (✉)
Institute of Bio-Sensing Technology, University of the West of England, Bristol, UK
e-mail: Vyv.Salisbury@uwe.ac.uk

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Abbreviations

AP	Alkaline phosphatase
AML	Acute myeloid leukemia
ara-C	Cytarabine, cytosine arabinoside
ara-CMP	Cytosine arabinoside monophosphate
ara-CTP	Cytosine arabinoside triphosphate
cdd	Cytidine deaminase
CLA	Cladarabine/cytarabine
DNR	Daunorubicin
dCK	Deoxycytidine kinase
FLA	Fludarabine/cytarabine
hENT1	Human equilibrative nucleoside transporter
IPTG	Isopropyl β-D-1-thiogalactopyranoside
NPM1	Nucleophosmin-1 gene
pyrE	Orotate phospho-ribosyltransferase gene

Contents

1 Introduction: Bioluminescent Biosensors for Drug Sensitivity 190
1.1 Bioluminescent Reporter Technology 191
1.2 Real-Time Monitoring of Anti-microbial Effects 191
1.3 Intracellular Anti-microbial Effects 192
2 The Clinical Need in Leukaemia 192
3 Construction of a Bacterial Biosensor for Chemotherapy Sensitivity Testing 198
3.1 Mode of Action of Target Drug 198
3.2 Construction of Cytarabine Sensitive *E. Coli* Host 198
3.3 Bioluminescent Biosensor Development 199
4 Validation of the Biosensor Assay EA 201
4.1 Validation of Ara-CTP Measurement 202
4.2 Assay Sensitivity and Specificity 203
4.3 Assay Reproducibility and Stability 204
5 Clinical Testing 204
6 Future Perspectives 207
6.1 Multi Drug Testing 209
6.2 Cytarabine Biosensor 210
6.3 Applications with Other Drugs 210
6.4 Additional Chemotherapy Biosensors 210
6.5 Rapid Testing in Other Cancers 211
References 211

1 Introduction: Bioluminescent Biosensors for Drug Sensitivity

The *luxCDABE* gene cassette from the terrestrial bacterium *Photorhabdus luminescens* has facilitated the wide range of clinical applications using bioluminescent reporters. In contrast to the marine bacteria *Aliivibrio fischeri* and *Vibrio harveii* where *lux* gene expression has a limited temperature range, the *lux* cassette from *P. luminescens* is fully functional at clinically relevant temperatures up to 45 °C [1] and can be inserted into Gram negative pathogens to enable rapid testing for antibiotic sensitivity and real-time monitoring of antimicrobial pharmacodynamics [2]. All the *lux* genes essential for luminescence are arranged in the single gene cassette; *luxCDE* encode a fatty acid reductase complex involved in synthesis of the fatty aldehyde substrate for the luminescence reaction catalysed by the luciferase *LuxAB* subunits [3] Two of the substrates for the bioluminescent reaction, reduced flavin mononucleotide ($FMNH_2$) and molecular oxygen, are both readily available in aerobic bacteria and expression of the complete *lux* cassette gives light output without the need for any exogenous substrate [1] The *P. luminescens lux* gene cassette has also been modified to give expression in Gram positive bacteria [4, 5] allowing extended clinical applications.

1.1 Bioluminescent Reporter Technology

Bioluminescent bacterial biosensors produce light as a direct indicator of the physiological status of the bacteria in real time. The high metabolic rate of bacterial cells, compared to mammalian cells, means that these reporters are ideal for fast, accurate, real time in situ testing. Response to drugs, toxic chemicals or other environmental insults can be measured in a non-destructive fashion with a high level of sensitivity and they are biosensors of choice for comparing the efficacies of fast acting biocides, within milliseconds of challenge [6]. Light output from the biosensor can be very accurately measured, with no background interference, using either luminometers or low light cameras.

There are some limitations to the employment of this technology; the need for molecular oxygen restricts the use of bioluminescent biosensors in anaerobic environments, although it has been reported that 10 nM oxygen is sufficient for luminescence detection [7]. Also the light produced by bioluminescent biosensors at a wavelength of 490 nM, is absorbed by mammalian cells and particularly by haemoglobin and melanin, with an estimated 10 fold reduction of light output for every cm of mammalian tissue [8]. However it is possible to image light output through skin muscle and bone—making non-invasive monitoring of disease processes possible using small mammals such as mice [9, 10]

1.2 Real-Time Monitoring of Anti-microbial Effects

The pharmacodynamics of antimicrobial agents are usually studied by observing changes in viable counts of bacteria over time for a range of drug concentrations, demonstrating the ability of the agent to prevent bacterial cell replication [11]. Although this method accurately demonstrates antimicrobial effects on bacterial cell replication, it is indirect and requires significant incubation time to produce countable bacterial colonies. Introduction of real-time monitoring using bioluminescent constructs of bacterial pathogens, where the *lux* genes are regulated by a constitutive promoter, has enabled direct, real time testing of antimicrobial effects on bacterial metabolism both in vitro and in vivo, using the neutropenic-mouse thigh model of infection [12].

A *Streptococcus pneumoniae* biosensor expressing the modified *P. luminescens luxABCDE* operon has been used to test novel antimicrobial agents including linezolid and gemifloxacin [4, 13]. Bioluminescence point readings and recovery counts were performed hourly over 12 h and showed that bioluminescence gave an earlier indicator of cellular recovery than counting techniques. Other fluoroquinolones, including moxifloxacin and ciprofloxacin, have been used to challenge a bioluminescent *E. coli* biosensor [2] and a bioluminescent biosensor strain of *Pseudomonas aeruginosa* [14], the latter in a continuous perfusion model of biofilm growth. Bioluminescent bacterial biosensors have also been used as an in vitro wound model for testing antimicrobial wound dressings [15] and a bioluminescent *E. coli* biosensor has demonstrated the antimicrobial properties of fresh human and bovine milk [16].

All of these bioluminescent bacterial biosensors were found to be sensitive, real-time reporters of antimicrobial efficacy. Similar biosensors have been used to assess susceptibility to fast acting biocides including phenol, Virkon and chlorhexidine digluconate [17], ethanol [18] and electrochemically activated solutions [6]. Bioluminescence may be used to monitor real-time, almost instant effects and distinguish the different mechanism of action of biocides.

1.3 Intracellular Anti-microbial Effects

Bioluminescent constructs of pathogenic bacteria have been successfully employed to monitor intracellular bacterial survival and antibiotic susceptibility inside mammalian cells [5]. Bioluminescent reporter bacteria are incubated with mammalian cells to allow for uptake. External bacteria are then removed with a bactericidal agent that is not taken up by mammalian cells (such as colistin or lysostaphin) and light output from an intracellular bioluminescent reporter can be used to monitor intracellular bacterial survival and drug penetration into cells.

Following on from this work—the possibility of using a bioluminescent bacterial biosensor for chemotherapy monitoring in leukaemic cells was considered.

The rapid, real-time monitoring of light output presented a simple and direct method of demonstrating drug uptake and conversion to an active form by leukaemic cells.

2 The Clinical Need in Leukaemia

Acute myeloid leukaemia (AML) is a heterogeneous group of haematological disorders resulting from the malignant transformation of myeloid precursor cells. The acute leukaemias are aggressive disorders in which one or more transformations occur in a haematopoietic stem cell or progenitor cell. This can lead to the development of a clonal malignancy via either increased proliferation, reduced cell death (apoptosis) or a block in the differentiation and maturation process. This leads to the proliferation of immature and undifferentiated cells in the blood and bone marrow and suppression of normal haemopoiesis [19]. The presenting symptoms are linked to bone marrow failure caused by accumulation of these malignant blast cells in the bone marrow, including frequent infections due to neutropaenia, anaemia and thrombocytopaenia (reduced clotting). In addition, infiltration of blasts cells may occur in the gums, skin and potentially central nervous system (CNS). If left untreated, the acute leukaemias are rapidly fatal, with patients succumbing within weeks or months. The disease is diagnosed by morphological examination of the blood and bone marrow, defined as the presence of >20 % blasts in the bone marrow at clinical presentation. Immunophenotyping is used to define the lineage of the leukaemic blasts and classify the disease according to the WHO classification of AML (2008). Accurate classification is very important as treatment selection and patient prognosis are intimately linked to the subtype of AML. In addition, much research has been dedicated to the investigation of key cytogenetic and molecular markers that are linked to prognosis and classification of the disease (Fig. 1). There is a clear correlation between cytogenetic scoring, categorised as favourable, intermediate or adverse and overall survival of AML patients [20]. One key mutation is the internal tandem duplication (ITD) of the *FLT3* gene, a receptor-type tyrosine kinase detected in approximately 20 % of AML patients and associated with a poor prognosis [21]. This effect can be negated by the presence of a point mutation in the nucleophosmin-1 gene *NPM1*, which reverses the poor prognosis for patients with *FLT3*-ITD [22] (Fig. 2).

One of the main issues for the patient is the speed of progress of the disease. When a patient is diagnosed with AML, they will often be required to commence treatment same-day. The decision to treat and the outcome for an individual patient will be dependent on a number of factors, with the single largest determinant being age of the patient at diagnosis. This is linked to the inability of elderly patients (>70 years) to tolerate the intensive chemotherapy regimens that have led to vast improvement in remission rates for younger patients (Fig. 3).

Given that the UK median age for diagnosis of AML is 65 years, this is a very important consideration. Other factors include sex of the patient, white blood cell

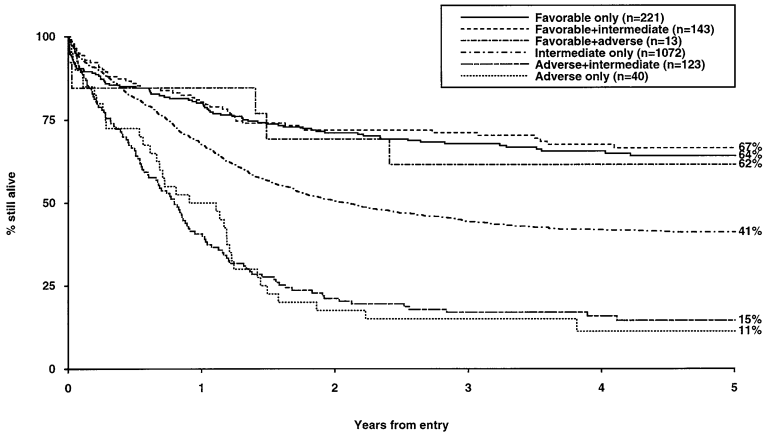

Fig. 1 Influence of cytogenetic abnormalities on overall survival in AML [2]

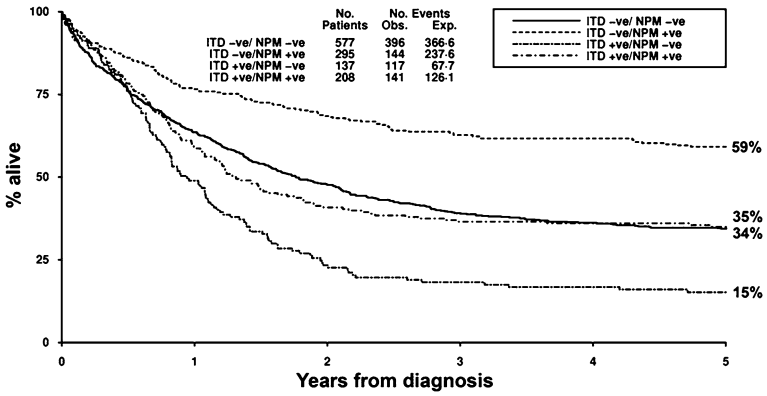

Fig. 2 Outcome stratified according to FLT3/ITD and NPM1 mutant status in total cohort [4]

(WBC) count at presentation, secondary disease status, and the specific genetic abnormalities associated with the patient's disease. Figure 4 illustrates patient progress following diagnosis. Treatment involves induction and consolidation phases to induce remission and to maintain low levels of any residual disease. Remission is defined as a reduction in the circulating blast burden by 95 % within the first 6 weeks post-commencement of treatment. The main chemotherapeutic agents used in induction and consolidation treatment of presenting patients are the anthracycline daunorubicin (DNR) and the nucleoside analogue cytosine arabinoside (cytarabine; ara-C).

Effective treatment of AML patients is still challenging and the clinical outcome is often unpredictable. Only 70 % of newly diagnosed patients receiving standard regimens including ara-C respond to treatment. Furthermore, a large

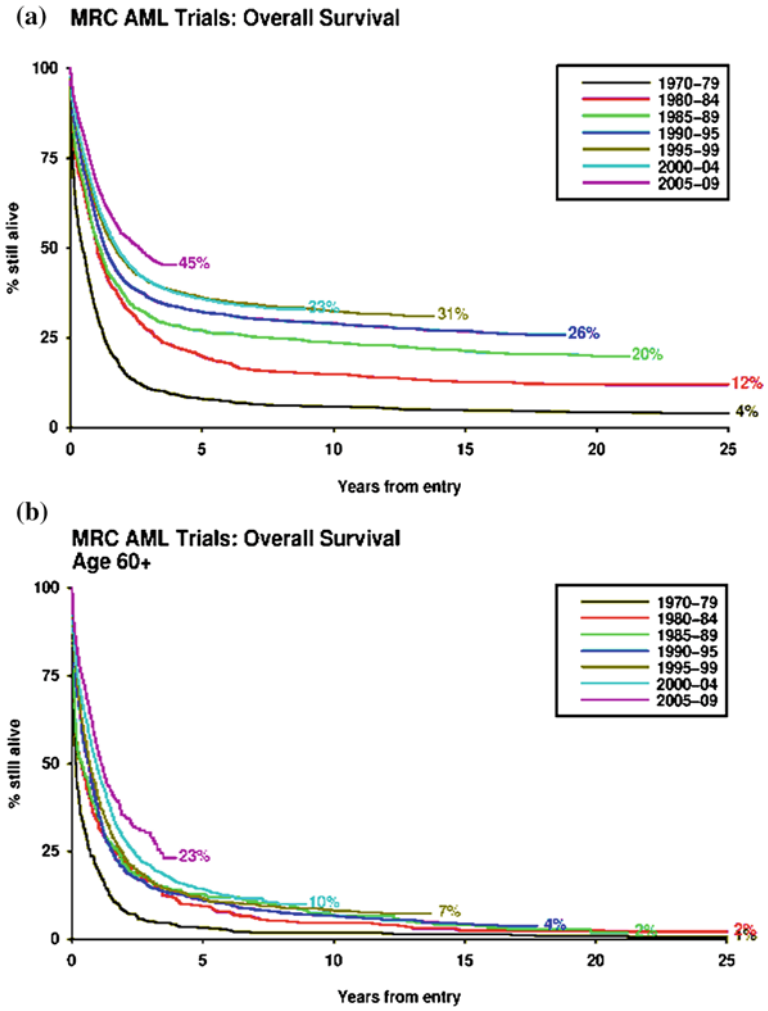

Fig. 3 Survival of adult patients with AML treated in UK trials over the past four decades. **a** Patients over 60 years and, **b** Aged 16–59 years

proportion of these patients fail to achieve long-term remission and also develop resistance to subsequent therapy [23].

Ara-C is one of the most active single anticancer agents and has been the mainstay treatment of AML for over three decades. It is used as in different doses at presentation (low-dose 20 mg/m^2/day, standard dose 200 mg/m^2/day or high dose 1.5–3 g/m^2/day) and in relapse where it is combined with other cytotoxic agents such as the ribonucleotide reductase inhibitor fludarabine [5]. Resistance to chemotherapy, including cytarabine, is a major reason for treatment failure among patients with AML [24]. A study from the Cancer and Leukaemia Group B

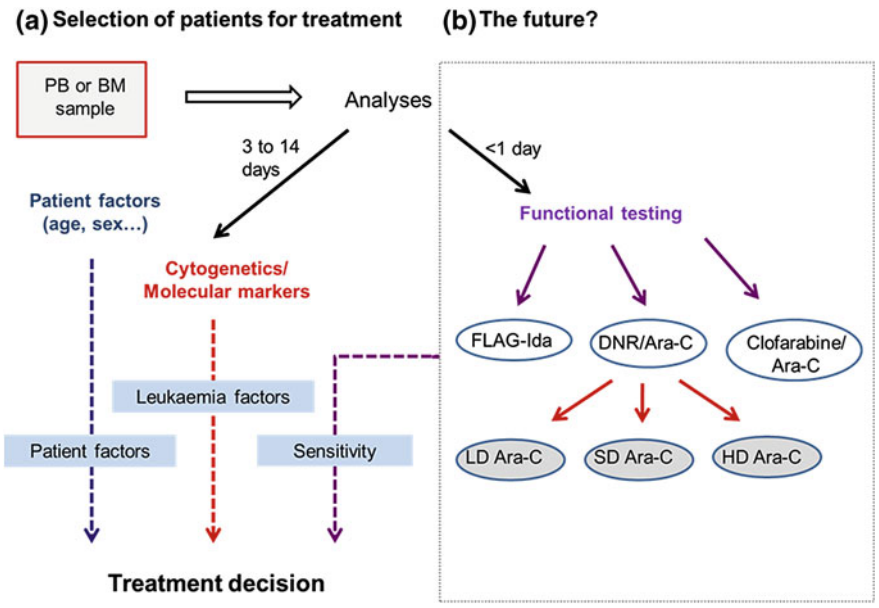

Fig. 4 Flow diagram illustrating **a** the current selection procedure for AML patients suitable for treatment with intensive chemotherapy and **b** the future including identification of the patient's individual chemosensitivity profile

examined 474 patients younger than 60 years and demonstrated a 34 % 5 year overall survival rate [25]. The outlook for older patients is even worse [26]. This is largely due to poor tolerability of intensive chemotherapy regimens in patients over 70 years, and the presence of primary disease resistance. Treatment-related mortality is a larger factor in the elderly, with death due to infection, haemorrhage and organ failure more common. Clinicians currently judge fitness of the individual patient, and if intensive treatment is deemed unsuitable then supportive care is provided with or without inclusion of low-dose single agent chemotherapy. Low-dose ara-C (20 mg/m^2/day) is used in this situation and can prolong survival without the morbidity and mortality associated with standard or high dose therapy in this age group.

In vivo, ara-C is transported into the cell via the specific human equilibrative nucleoside transporter (hENT1), and is rapidly phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form. Ara-CMP is further phosphorylated by nucleoside kinases into its active tri-phosphorylated form, ara-CTP. Drug inactivation can result from ara-C conversion into ara-uracil (ara-U) by cytidine deaminase or from dephosphorylation of ara-CMP by cytoplasmic nucleotidase [27]. The antiproliferative and cytotoxic effects of ara-CTP are due to its ability to interfere with DNA polymerase and to incorporate into DNA strands leading to chain termination and DNA synthesis arrest. High-dose ara-C can also cause accumulation of cytochrome c in the cytosol, loss of mitochondrial membrane

potential and an increase in reactive oxygen species [24, 25]. Ara-C has recently been found to induce apoptosis via the death receptor pathway, involving signalling through sphingomyelin enriched plasma membrane lipid rafts [26]. Chemoresistance to ara-C can arise from a number of factors influencing the rate of ara-CTP formation and incorporation into DNA including low drug uptake via reduced expression of the transporter hENT1, increased conversion into ara-U by cytidine deaminase, reduced level or activity of the enzyme dCK, or increased dephosphorylation of the active metabolite by cytoplasmic nucleotidase [28].

AML is treated at presentation with a combination of DNR and ara-C. DNR inhibits topoisomerase II and subsequent uncoiling of DNA prior to DNA replication, and is also used in combined chemotherapy regimens to treat acute lymphoid leukaemia (ALL) and non-Hodgkin's lymphoma. DNR is a chemotherapeutic drug with serious adverse effects including cardiotoxicity that impacts on the usefulness of the drug. This cardiotoxicity is cumulative and so treatment with DNR at presentation precludes use of the drug after induction failure. For those patients that fail to achieve remission or who subsequently relapse a second cocktail of chemotherapeutic agents is used. The nature of AML means that the relapsed clone is often immunophenotypically distinct from the presenting clone and will require an alternative treatment regimen. At relapse the most common regimen is fludarabine, high dose ara-C, the anthracycline idarubicin and granulocyte-macrophage colony stimulating factor (GM-CSF). Fludarabine, and more recently clofarabine, are purine analogues that inhibit ribonucleoside reductase in the AML cell, reducing the ability of the cell to produce endogenous nucleotides. This acts to potentiate the intercalation of ara-CTP into the DNA, and can be very useful in treating cells that do not respond well to ara-C alone.

It is important to emphasise that currently no pre-treatment assessment of chemotherapy efficacy is performed for individual patients prior to treatment. In the research setting in vitro assessment of ara-C efficacy has traditionally involved measurement of (a) cell death (b) reduction in S-phase activity or (c) use of AML clonogenic assays for leukaemic cells exposed to ara-C [29]. However, these methods are non-standardised, time consuming, expensive and are not suitable for routine screening. Therefore, patients are treated with regimens including ara-C regardless of their sensitivity to the drug and can suffer the serious side-effects such as myelosupression, chemical conjunctivitis, cerebellar dysfunction and the development of drug resistant secondary cancers [30].

The use of bioluminescent biosensor technology to develop ara-C/DNR and ara-C/fludarabine biosensors has great potential to make a difference to patients with AML. A biosensor to predict response to anthracyclines (daunorubicin, doxorubicin, idarubicin) would have particular benefits because they cause dose-dependent cardiotoxicity that can lead to clinical heart failure. For example, if the biosensor predicts a high level of anthracycline sensitivity, treatment with reduced dose lessens risk of heart damage. Prediction of resistance may lead a clinician to avoid this cardiac-associated risk altogether in favour of an alternative combination therapy.

Patients with AML and their healthcare teams, including clinicians, laboratory workers and Acute Healthcare Trusts could benefit directly and immediately.

Patients could benefit from receiving tailored treatment that will specifically target their cancer, providing the clinician with a 'drug response profile' to combinations and doses of chemotherapeutics, to inform patient treatment decision making. Patients with highly responsive cancer cells (especially those over 70 years) could be given effective low dose chemotherapy with subsequent reduced side effects, including reduction in hospitalisation, time off work and risk of secondary malignancies. The rising numbers of elderly AML patients will be key beneficiaries. These patients may have health conditions that rule out aggressive chemotherapy; test results that indicate effectiveness of low dose chemotherapy will be vital for their treatment. Those with ara-C resistant cells could be given effective combined chemotherapy to negate the risks of single agent failure.

Patients will also benefit from receiving test results in the first 24 h of diagnosis, a time of maximum anxiety for patients and their families. Rapid results that indicate the most effective chemotherapy will help with difficult decisions about treatment that have to be made within hours of diagnosis for this rapidly progressive disease.

Healthcare providers will benefit from targeted chemotherapy leading to reduced hospitalisation of patients with adverse side effects and possible reduction in additional courses of chemotherapy. In the long term, the bioluminescent biosensor technology could be used to predict response to chemotherapy in a range of haematological malignancies, including the chronic leukeamias, lymphomas and myelomas and also in solid tumours, where minor adjustments of the assay protocol would allow rapid testing of biopsy material for response to an array of relevant drugs. This is a platform technology; the biosensor is capable of response to a number of cytotoxic nucleoside analogues and could also be used for therapeutic drug monitoring. The technology has the potential to contribute to the health of patients diagnosed with haematological and solid tumour cancers by rapidly predicting response to different doses and combinations of chemotherapy.

3 Construction of a Bacterial Biosensor for Chemotherapy Sensitivity Testing

3.1 Mode of Action of Target Drug

Cytarabine (ara-C) is an anti-cancer agent widely used in chemotherapy for over three decades. In vivo, ara-C is transported into the cell via the specific nucleoside transporter (hENT1), and is rapidly phosphorylated by deoxycytidine kinase (dCK) to its monophosphate form. Ara-CMP is further phosphorylated by nucleoside kinases into its tri-phosphorylated active form ara-CTP [31]. Drug inactivation can result from ara-C conversion into ara-uracil by cytidine deaminase or from dephosphorylation of ara-CMP by cytoplasmic nucleotidase [29]. The antiproliferative and cytotoxic effects of ara-CTP are due to its ability to interfere with

DNA polymerase and to incorporate into DNA strands leading to chain termination and DNA synthesis arrest [32]. Resistance to ara-C results from factors that influence the rate of ara-CTP formation and incorporation into DNA; a biosensor that measures ara-CTP levels within leukaemic cells will predict sensitivity or resistance to the drug [31].

In vitro assessment of ara-C efficacy has traditionally involved measurement of leukaemic cell death or S phase activity, following exposure to the drug, or the use of clonogenic proliferative assays [29]. These methods are cumbersome, time consuming, expensive and unsuitable for routine screening. Therefore patients are treated with regimens including ara-C regardless of their sensitivity to the drug. [31].

3.2 Construction of Cytarabine Sensitive E. Coli *Host*

Ara-C has no effect on *E. coli* as the bacteria lack deoxycytidine kinase (dCK) and deaminate ara-C into ara-uracil through the activity of deoxycytidine deaminase (cdd). Alloush et al. [31] constructed a suitable cytarabine sensitive strain by starting with *E. coli* MG1655, an auxotrophic derivative of wild-type *E. coli* K-12 that requires pyrimidine in order to grow in minimal medium, due to suboptimal expression of the enzyme orotate phospho-ribosyltransferase coded for by the orotate phosphoribosyltransferase (*pyrE*) gene [34]. This strain was rendered cdd-deficient using random transposition mutagenesis with P1phage carrying a Tn10 transposon, which can transpose from the phage into the *E. coli* chromosome. Mutants carrying the transposon were selected on Luria Bertani agar plates containing 10 mg/L tetracycline. The *cdd* mutants were selected after 24-h incubation by growth at 37 °C on plates containing10 mg/L of the analog 5-fluoro-2_ deoxycytidine, which is toxic to *E. coli* expressing *cdd* activity [31]. The resulting *pyrE* deficient, *cdd* deficient strain was transformed with plasmid pTrcHUMdCK, expressing the human deoxycitidine kinase cDNA under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter [35]. The transformed strain gave IPTG inducible expression of dCK resulting in growth inhibition by 100 μM ara-C, as long as IPTG was present (Fig. 5b). This constituted a suitable host bacterial strain [31], with increased uptake of ara-CTP, rather than de novo dCTP synthesis (due to low level of *pyrE* expression), reduced conversion of ara-C to ara-U (due to *cdd* transposon knockout) and increased conversion of ara-C to ara-CTP (due to IPTG inducible dCK expression).

3.3 Bioluminescent Biosensor Development

The broad host range vector plasmid pBBR1MCS-2 [36] carrying the *luxCDABE* cassette from *P. luminescens* as an *Eco*R1 PCR fragment, was used by Alloush et al. [31] to transform the cytarabine sensitive host *E. coli* strain to give the self

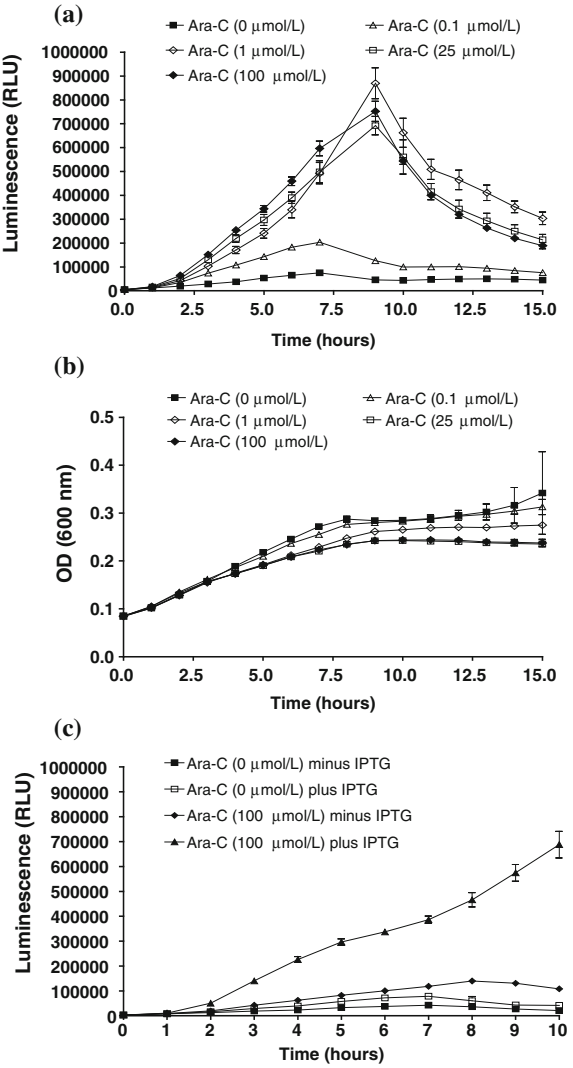

Fig. 5 Biosensor response. The effect of ara-C concentrations (0.1, 1, 25, and 100 µmol/L) on luminescence (**a**) and growth (**b**) of *E. coli* HA1 in the presence of IPTG; the effect of IPTG induction of dCK in the presence of ara-C (**c**) is also shown. Growth was monitored by measuring 600 A; luminescence is shown as relative light units (RLU); n = 10 and error bars represent SD

bioluminescent bacteria biosensor *E. coli* HA1. When challenged in LB broth or RPMI 1640 medium with ara-C in the presence of IPTG, there was a significant increase of light output from the biosensor (Fig. 5a) with concentrations as low as 0.1 µmol/L. This significantly increased light output was only observed during treatment with the pyrimidine analog ara-C and only in the presence of IPTG-activated dCK (Fig. 5c). The specificity of the biosensor to ara-C was indicated by a lack of biosensor response in a control assay with the purine analog fludarabine. To increase the specificity of the biosensor, direct effects of the active intracellular drug derivative, ara-CTP, on the bacterial biosensor were monitored (Fig. 6). The

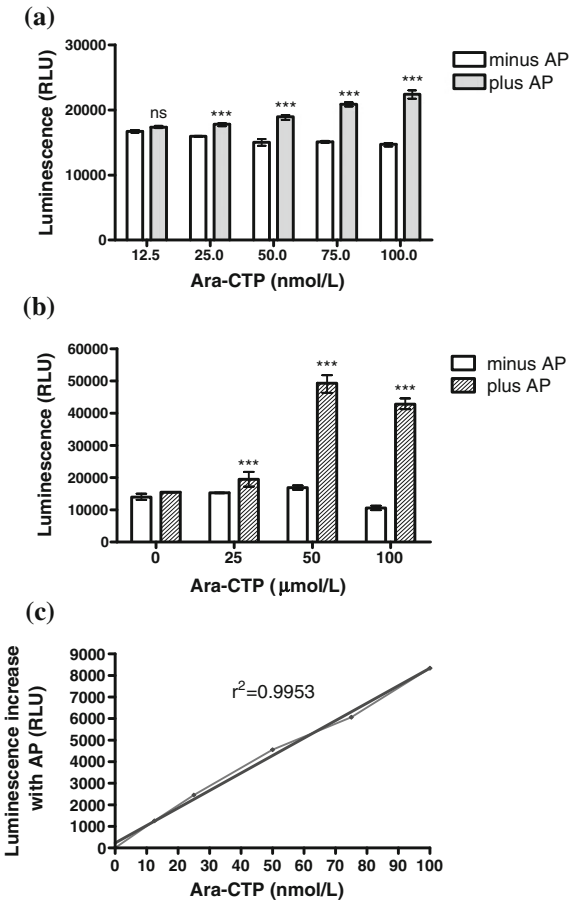

Fig. 6 The effect of ara-CTP on the biosensor. *Peak* light emission, measured as relative light units (RLU), from *E. coli* HA1 in RPMI at 37 °C treated with ara-CTP at 12.5, 25, 50, 75, and 100 nmol/L (**a**) and 0, 25, 50, and 100 µmol/L (**b**) in the absence and presence of AP (mean of n = 3 and error *bars* show range). Correlation between ara-CTP and light emission ±AP (**c**)

results indicate that ara-CTP does not enter the reporter bacteria, showing no increase in light output compared with the untreated control, unless alkaline phosphatase is added at the start of the assay. The increase in peak light output in the AP-treated samples (Fig. 6a, b) is similar to that observed with ara-C [31].

The bioluminescence increase brought about by ara-C in the biosensor is similar to previous reports of enhanced light emission in luciferase-based biosensors brought about by impairment of the bacterial intracellular equilibrium, leading to NADPH accumulation [37], or by DNA damage [38]. It is reported that bioluminescence stimulates DNA repair in bacteria, possibly by providing photons for bacterial photolyase activity [39] and that lux genes are regulated by the bacterial SOS stress response [40], which may explain the increase in bioluminescence in the presence of ara-C.

The bioluminescent bacterial biosensor developed by Alloush et al. [31] can be maintained stably by selection on nutrient agar supplemented with 10 mg/L

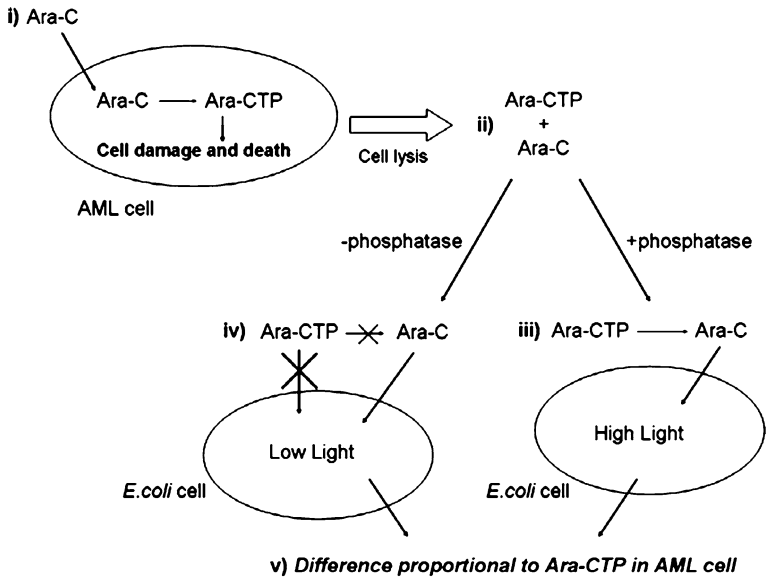

Fig. 7 Biosensor assay procedure. *(i)* AML cells from patients with AML exposed to *ara-C* are washed and lysed in the presence of EDTA and saponin. *(ii)* Subsequent cell lysate (containing both *ara-C* and *ara-CTP*) are exposed to the bacterial biosensor in the presence or absence of alkaline phosphatase (*AP*). *(iii)* In the presence of *AP*, *ara-CTP* is converted to *ara-C*, which on entering the bacterium allows the generation of bioluminescence. *(iv)* In the absence of *AP ara-CTP* remains intact and cannot enter the bacterium. *(v)* The ratio between ±AP is directly proportional to the concentration of *ara-CTP* in the patient blasts, which is representative of the patient's ability to convert *ara-C* to *ara-CTP*

tetracycline, 100 mg/L ampicillin and 25 mg/L kanamycin (for maintenance of Tn10, pTrcHUMdCK and pBBR1MCS-2lux+, respectively). It can be lyophilized for use in a routine in vitro assay for cytarabine sensitivity of clinical samples of leukaemia patient bone marrow or peripheral blood.

4 Validation of the Biosensor Assay EA

Following design and construction of a biosensor with enhanced sensitivity to ara-C, we developed the technology into a rapid assay to determine ara-C uptake and phosphorylation by human leukaemic cells (Fig. 7). The assay principle involves exposure of AML blast cells to ara-C (25 μM) for 30 min, during which a proportion of ara-C is transported into the cell via the transporter hENT1, and metabolised by dCK to ara-CTP. Following exposure to ara-C, blasts are washed and lysed by addition of saponin (which does not affect the integrity of the bacterial biosensor) in the presence of EDTA to inhibit endogenous alkaline

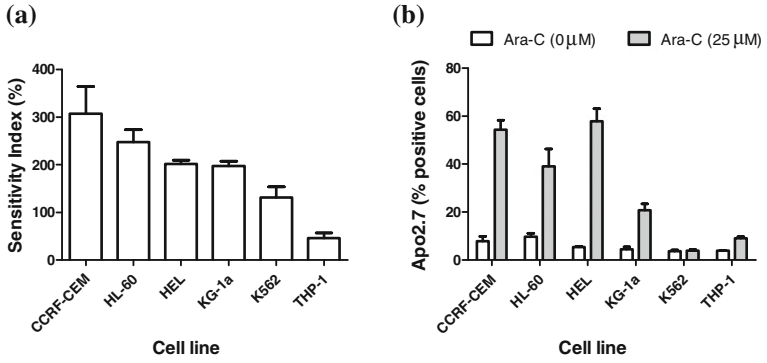

Fig. 8 Comparison of **a** accumulation of ara-CTP in leukaemic cells as measured by the biosensor assay after 30 min incubation with ara-C and **b** expression of the apoptotic marker APO2.7 in cells treated for 48 h with ara-C. APO2.7 detects a mitochondrial protein, 7A6 that is expressed in early apoptosis

phosphatases (AP). The addition of exogenous AP results in conversion of ara-CTP to ara-C that can enter the biosensor and induce bioluminescence. Without addition of AP only ara-C can enter the biosensor, and the remaining pool of ara-CTP remains excluded. The resultant difference in bioluminescence is proportional to the level of ara-CTP in the lysate.

4.1 Validation of Ara-CTP Measurement

Validation of this biosensor assay was carried out using immortalised leukaemic cell lines with known and differing sensitivities to ara-C [41]. Cell lines were treated in vitro with ara-C at a range of in vivo relevant concentrations, and the effect assessed using a range of research techniques. Results from the biosensor assay were correlated with measurement of intracellular concentration of ara-CTP by reverse phase high performance liquid chromatography analysis (HPLC), APO2.7 staining of apoptotic cells, and assessment of cell death using a commercially available 3-day in vitro assay for assessment of cellular viable mass.

Across the cell lines treated with cytarabine (25 μM) there was a significant correlation between ara-CTP levels measured using HPLC and the sensitivity index values from the biosensor assay ($R = 0.9722$, $p = 0.0028$) indicating that the biosensor is accurately measuring intracellular ara-CTP. In addition, there was a correlation in the majority of the cell lines between ara-CTP accumulation and cell death as measured by 3-day cytotoxicity assay, and expression of the mitochondrial protein 7A6, expressed in early apoptosis (Fig. 8). This indicates that the biosensor assay is capable of determining relative sensitivity to ara-C.

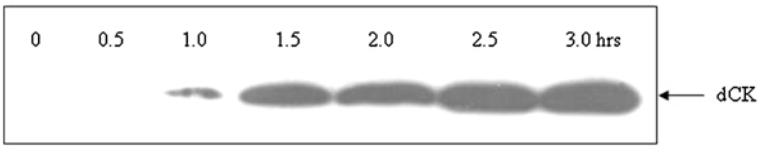

Fig. 9 Representative Western blot for time course of IPTG-induced expression of dCK protein between 0 and 3 h

4.2 Assay Sensitivity and Specificity

Figure 9 shows expression of the human *dCK* gene within the biosensor. This is selectively controlled by addition of IPTG to the biosensor, which causes a time-dependent increase in the level of dCK as measured by Western Blotting using a specific antibody. This corroborated by a time-dependent increase in light output from the biosensor in response to ara-C (Fig. 10a) that is specific to ara-C and not the purine analogue fludarabine (F-ara-A) (Fig. 10b).

Figure 11 illustrates the inverse relationship between light output from the biosensor and toxicity of ara-C to the biosensor strain. On addition of ara-C the biosensor demonstrates an impressive 2.5-log increase in light output per bacterial colony-forming unit (cfu) (Fig. 10a). This is emphasised by the concurrent decrease in bacterial viability in response to ara-C (Fig. 10b).

Figure 12a, b describe the raw data and resultant calibration curve produced using the concentration of spiked ara-CTP (0–0.5 μM) against bioluminescence ± exogenous AP results from the biosensor ($r^2 = 0.997$). The limit of detection of ara-CTP using the biosensor was found to be 0.025 μM and a linear relationship was observed up to 0.5 μM. This concentration range represents a suitable detection range to allow differentiation between ara-C responsive and non-responsive leukaemic cell lines.

4.3 Assay Reproducibility and Stability

To evaluate assay precision [42], we measured the effect of three calibrators of known ara-CTP concentration (0.05–0.5 μM) each repeated three times on subsequent days (n = 5/experiment) (Table 1). Standard deviation (SD) and coefficient of variation (CV) were calculated for intra-day and inter-day assay results. An acceptable level of precision was defined as ±15 % CV, which was achieved for the LQC, MQC and HQC samples (0.05–0.5 μM). A 5-point calibration curve was employed in parallel for each run (0–1 μM), and SI % values were back-calculated to provide concentrations for the QC results. Intra- and inter-run CV <15 % were achieved for all QC levels across the three runs.

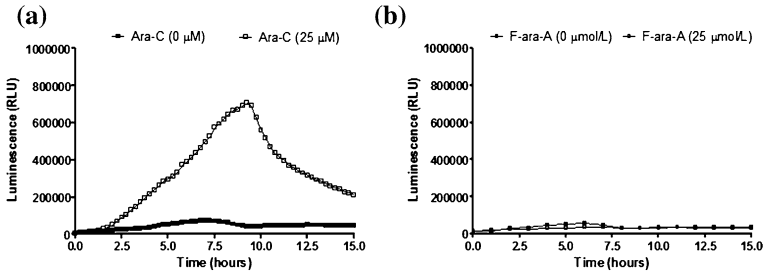

Fig. 10 The effect of IPTG-induced expression of dCK on light emission from the biosensor following exposure to the pyrimidine analogue ara-C (**a**) but not the purine analogue fludarabine (F-ara-A) (**b**). RLU, relative light units

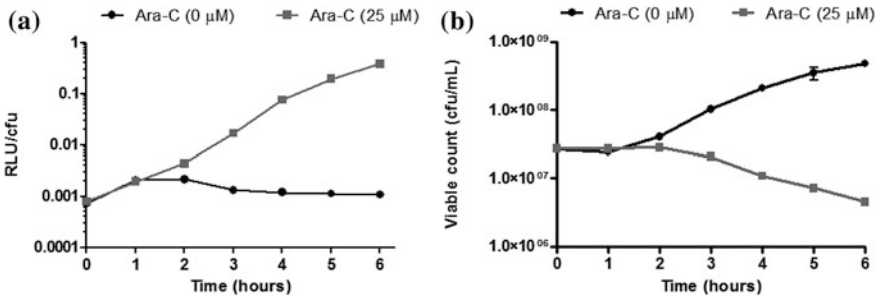

Fig. 11 The relationship between light output and toxicity of ara-C to the biosensor strain. **a** Light output from the biosensor per colony-forming unit (*cfu*) in response to ara-C, and **b** the effect of ara-C on growth of the biosensor. *RLU* relative light units

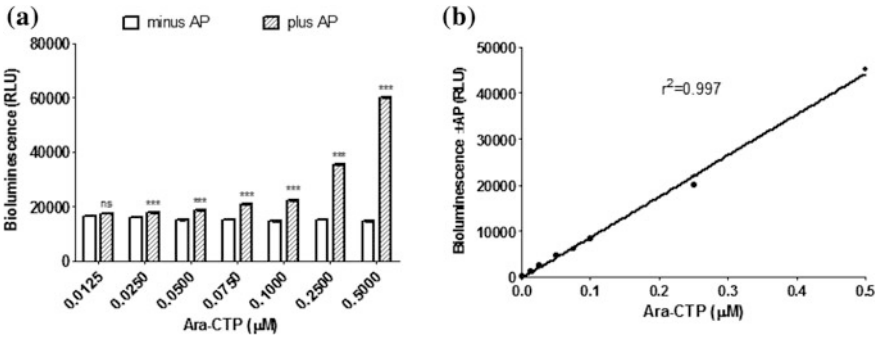

Fig. 12 *Peak* bioluminescence from the biosensor treated with ara-CTP (0–0.5 µM) ± alkaline phosphatase (**a**), and the resultant calibration *curve* (**b**)

For convenience and ease of use in a clinic laboratory setting, the biosensor was lyophilized and the stability verified over a 12 month period. Figure 13 describes the results for (a) inter-batch (batches #1–5) and (b) intra-batch (batch 5) stability of the lyophilized biosensor. In all cases, lyophilized calibrators containing

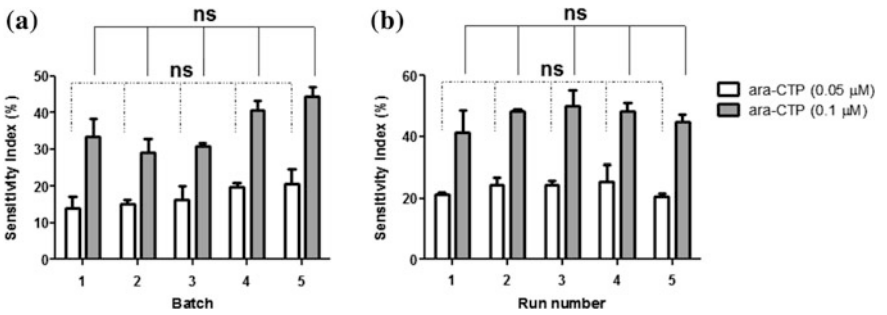

Fig. 13 Inter- and intra-batch stability testing for the lyophilized biosensor. **a** Inter-batch assessment was performed using 5 batches of lyophilized biosensor produced over a period of 12 months. **b** Intra-batch assessment was performed using batch 5 of the lyophilized biosensor over the period of 12 months (n = 5)

Table 1 Assessment of assay precision using QC samples assayed in three independent batch runs

Run id	LQC (0.05 μM)	MQC (0.1 μM)	HQC (0.5 μM)
1			
Intraday mean	0.049	0.112	0.451
Intraday SD	0.006	0.011	0.050
Intraday CV %	11.5	10.0	11.0
2			
Intraday mean	0.045	0.087	0.471
Intraday SD	0.005	0.012	0.048
Intraday CV %	12.0	13.5	10.1
3			
Intraday mean	0.050	0.112	0.426
Intraday SD	0.007	0.015	0.037
Intraday CV %	14.1	13.4	8.8
Mean result	0.048	0.103	0.449
Interday SD	0.003	0.015	0.022
Interday CV %	5.5	14.2	5.0
n	15	15	15

Three levels of QC sample were produced covering a range of ara-CTP concentrations of clinical relevance (0.05–0.5 μM). A 5-point calibration curve was used to back-calculate the concentration of ara-CTP in each sample and the mean calculated within run (n = 5). Intra-day and inter-day standard deviation (*SD*) and coefficient of variation (*CV* %) were calculated

ara-CTP in blank sample matrix were used, termed low (0.05 μM ara-CTP) and medium (0.1 μM ara-CTP) calibrators. Batch-to-batch mean SI % values from the biosensor for low and medium calibrators were 14.8 and 31.9 %, with acceptable CV % values (<15 %) for both batch-to-batch and run-to-run analysis (n = 5). Longer-term and accelerated stability studies are currently on-going to ensure maintenance of assay characteristics.

5 Clinical Testing

Initial clinical testing of the biosensor assay used fresh peripheral blood and cryopreserved bone marrow samples. Mononuclear cells from fresh AML peripheral blood samples (collected at Frimley Park Hospital, Surrey, UK from patients following informed consent) were isolated by density gradient centrifugation and re-suspended in RPMI 1640 medium. Cryopreserved samples, previously separated by density gradient centrifugation to yield the mononuclear cell fraction, were thawed and re-suspended in RPMI 1640 medium prior to incubation with ara-C. In all cases, samples were obtained from patients at presentation with blast burdens >80 % for whom clinical outcome following induction therapy with ara-C was known. In vitro dosing was performed with ara-C at 25 μM, which represents the equivalent of the standard in vivo dose of 200 mg/m^2/day (based on an 80 kg individual).

Preliminary testing of these blood and bone marrow samples taken at presentation from 12 known responding and 12 known non-responding ara-C–treated patients gave biosensor assay responses between 21 and 128 % (median 55 %) for responding patients and between −7 and 6 % (median 0 %) for non-responding patients (Fig. 14). Assay precision is highest at extremes of the detection range (Table 1), indicating that the assay should perform well in a clinical setting. A typical example of each response type is shown in Fig. 15; patient sample CR (with clinical outcome of complete remission) showed a significant difference in the peak light output ($P < 0.001$) in the presence and absence of AP, indicating response to a drug (Fig. 15a), whereas sample NR (nonresponsive clinical outcome) exhibited no significant difference ($P > 0.05$) in light output between the (±) curves, indicating a low concentration of ara-CTP in the cells (Fig. 15b).

In theory any agent capable of potentiating generation of ara-CTP from ara-C can also be tested on the biosensor system. Figure 16 shows results using the assay for combination therapy screening in a patient that received induction therapy with DNR and ara-C but who subsequently relapsed and was treated with a regimen containing fludarabine/ara-C (FLA). The biosensor assay was performed on this sample with ara-C alone or in conjunction with the purine analogue fludarabine. Figure 16 shows an increase in light from the ara-C-treated sample +AP, however pre-treatment with fludarabine (highlighted in red) induced a significant increase in light, indicating that fludarabine had potentiated generation of ara-CTP in this patient.

The biosensor assay was optimised for this combination therapy using a range of pre-incubation periods based on the protocol for in vitro dosing proposed by Ahlman et al. [43]. The optimal dosing schedule required 4 h pre-incubation with fludarabine (5 μM) followed by the optimised protocol with ara-C dosing. This biosensor assay system offers a valuable tool in predicting response in patients receiving ara-C or fludarabine/ara-C. This may be of importance in patients demonstrating chemoresistance, at presentation and at relapse.

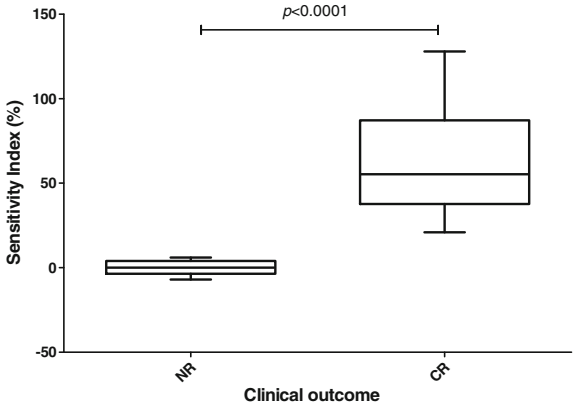

Fig. 14 Box-Whisker plot of patient data from responders (n = 12) and non-responder (n = 12) to ara-C treatment. Median results were 55 and 0 % respectively. *NR* non-remission, *CR* complete remission

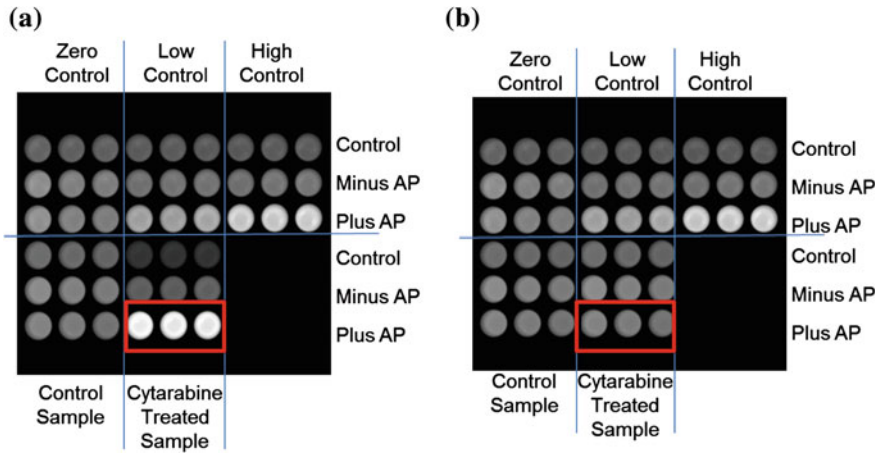

Fig. 15 Comparison of results from the biosensor for (**a**) a patient that responded to treatment with a regimen including ara-C after one cycle of chemotherapy, and (**b**) a patient that failed to respond to ara-C. Areas highlighted in *red* represent ara-C treated samples incubated with exogenous alkaline phosphatase (*AP*) and show increased light in (**a**) but not in (**b**) indicating that patient **a** was capable of producing sufficient ara-CTP to induce remission, whereas patient **b** was not. Controls with known quantities of ara-CTP (zero, low and high) are included to quality control the results. **a** Sensitive patient (remission after 1st cycle). **b** Resistant patient (no remission)

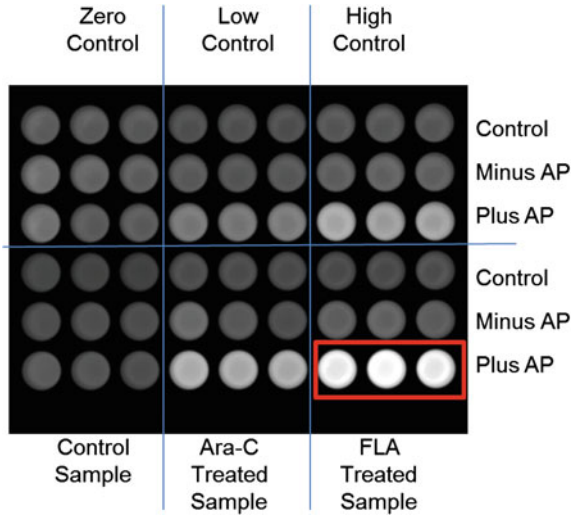

Fig. 16 Comparison of results from the biosensor for a patient that failed to respond to DNR/ara-C at presentation but who responded to treatment with a regimen including fludarabine and ara-C (FLA) at relapse. Light from the biosensor increased dramatically following pre-treatment with fludarabine (*red box*) compared to ara-C alone, indicating that fludarabine was capable of potentiating the production of sufficient ara-CTP to induce a response. *Controls* with known quantities of ara-CTP (zero, low and high) are included to quality control the results

6 Future Perspectives

The construction of a bioluminescent bacterial biosensor for chemotherapy sensitivity testing has paved the way for a rapid assay platform that can be used in a clinical setting, enabling determination of the degree of response before patients undergo chemotherapy.

A single drug predictive test has been designed and validated, for use before AML patients start ara-C chemotherapy. This test has shown close correlation with (i) a 3-day commercially available cytotoxicity test (Promega CellTiterGlo®) and (ii) patient clinical outcomes to therapy. The same blood samples could be concurrently tested for response to combined chemotherapy. Using this technology to develop ara-C/DNR biosensors has great potential. This would be invaluable for patients with AML that frequently require same-day diagnosis and commencement of treatment. At present there is no simple, same-day technology on the market for screening AML patient samples before starting a course of single or combined chemotherapy and no test showing development of resistance during treatment. Also, for patients with very responsive cancer, there is no predictive test to indicate effective minimally toxic treatment with low dose chemotherapy.

6.1 Multi Drug Testing

A biosensor to predict response to anthracyclines (daunorubicin, doxorubicin, idarubicin) would have particular benefits because they cause dose-dependent cardiotoxicity that can lead to clinical heart failure. If the multi-drug test device predicts a high level of anthracycline sensitivity, treatment with reduced dose lessens risk of heart damage. Prediction of resistance may lead a clinician to avoid this cardiac-associated risk altogether in favour of an alternative combination therapy. A multi-drug test device may also have use after the first round of chemotherapy, if patients develop resistance to ara-C. Initial testing with fludarabine/ara-C (Fig. 5.3) and a range of analogues of fludarabine has shown that the biosensor could be used to predict the response to treatment for an individual with relapsed AML.

Current research focuses on miniaturisation of the test to enable more drugs and doses thereof to be tested using a single peripheral blood sample or bone aspirate. The further projected scale of the market for this chemotherapy theranostic biosensor is substantial, because it has the potential to be developed to screen for sensitivity to a wide range of anti-cancer drugs and also, with modification, could be extended to solid tumour biopsy material.

A quick and simple multi-drug test device is under development, based on blood or bone marrow samples before the start of treatment, to show response to combined drugs used for treatment. The results of the multi-drug test will ensure that a patient receives the right combination of chemotherapy drugs, in the correct dose, to meet their needs and so prevent any delay in effective treatment. The test device will be particularly useful to patients whose leukaemic cells are sensitive to low doses of chemotherapy, allowing them to be treated with minimum drug doses, with less side effects. For elderly patients, where AML incidence and mortality rates are increasing, and where other health conditions may rule out aggressive chemotherapy, this screening test could be particularly timely.

6.2 Cytarabine Biosensor

The current bacterial biosensor relies on gene expression from two plasmids, *pTrcHUMdCK*, expressing the human deoxycytidine kinase cDNA under the control of an IPTG-inducible promoter and *pBBR1MCS-2lux+*, expression the *lux* genes for self bioluminescence. Although this biosensor is relatively stable provided that antibiotic selection for the plasmids is maintained during growth and lyophilisation, it could be an advantage to insert the human *dCK* gene and the *luxCDABE* genes into the chromosome of the *E. coli* host.

6.3 Applications with Other Drugs

Preliminary screening indicates that the *E. coli* HA1 biosensor strain can be used to determine resistance or sensitivity to deoxyribonucleoside kinase-dependent drugs, i.e. nucleoside analogue drugs that utilise a deoxyribonucleoside kinase (dNK) pathway. This includes cladribine, used in treatment for hairy cell leukaemia and multiple sclerosis; gemcitabine, used in pancreatic, breast and non-small cell lung cancers; nelarabine, used for T-cell acute lymphoblastic leukaemia; zalcitabine, lamivudine and zidovudine used in HIV treatment and acyclovir used for Herpes virus. The biosensor has potential use both for sensitivity screening and therapeutic drug monitoring of these drugs.

6.4 Additional Chemotherapy Biosensors

Development of the biosensor and assay protocol has opened up the field for additional bioluminescent bacterial biosensors that respond to widely used chemotherapeutic drugs.

Anthracyclins, including daunorubicin, doxorubicin and idarubicin, remain an important class of chemotherapeutic agents. However, their efficacy in treating cancer is limited by a cumulative dose-dependent cardiotoxicity, which can cause irreversible heart failure [44]. Construction and evaluation of a bioluminescent biosensor to rapidly monitor drug sensitivity of cancer cells in clinical samples will be of considerable use to inform dosing strategies with these drugs.

6.5 Rapid Testing in Other Cancers

The need for rapid testing in haematological malignancies such as AML is clearly established. However the technology could be extended to solid tumours, where biopsy samples could be treated for disaggregation before undergoing rapid assay with bioluminescent biosensors. It could be of use in brain tumours where chemotherapy may be the only course of treatment and also in breast and other tumours where treatment with chemotherapy is used to reduce tumour size before removal.

It is clear that in the field of predicting response of cancer cells to chemotherapy, there have been great advances in genotypic testing and there many rapid tests for marker genes available to clinicians. But some malignancies, including AML are known to have heterogeneous origins and the possibility of employing bioluminescent bacterial biosensors for rapid testing of the phenotypic response to cancer cells to chemotherapeutic drugs has been welcomed by clinicians. It is hoped that it will be a contributory factor in a movement towards stratified treatment for individual patients, dependent on in vitro response of their tumour cells.

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Index

0-9
1-methyl-3-nitro-1-nitrosoguanidine, 139
2-(2-naphthalenyl)benzothiophene, 105
2,4 dinitrotuloene (2,4-DNT) bioreporter, 141, 142
  dose-dependent fluorescence response of, 146*f*
2,4-dichlorophenol (DCP), 78, 79, 80, 88
  Burkholderia sp. RASC c2, 85, 94*t*
3,5-dichlorophenol (DCP), 90*t*, 96*t*, 101
5′-fluoroluciferin analogue, 46
6th Water Framework Directive, 105

A
Acinetobacter calcoaceticus, 145
Acinetobacter sp. DF4/pUTK2, 85–86, 94*t*
Acute lymphoid leukaemia (ALL), 197, 211
Acute myeloid leukaemia (AML), 193, 210, 211, *see also* Leukaemia, clinical need
  ara-C, 195, 196, *see also* Cytarabine (Ara-C)
  bioluminescent biosensor technology, 197
  biosensor assay procedure, 202*f*
  clinical testing, 207
  DNR and ara-C, combination of, 197
  drug response profile, 198
  influence of cytogenetic abnormalities, 194*f*
  selection procedure for treatment, 196*f*
  survival of adult patients, 195*f*
Acute toxicity
  assays, 76, 110
  chronic toxicity based on, 100
  E. coli HB101 (pUCD607), in measuring, 77
  tests
    bioluminescence tests, 115
    Microtox™ method, 70
Adenosine diphosphate (ADP), 35–36, 37, 47, 48, 49, 50, 57, 58
Adenosine monophosphate (AMP), 32, 35, 37, 47, 48, 50, 58
  luciferase-oxyluciferin—AMP complex, 43
Adenosine triphosphate (ATP), 22, 32, 138, 174*t*
  assays, 38–39, 54
    ATP depletion, 51–52, 55, 56
    ATP formation, 49–51, 55
  in bacterial cells, 35
  contaminated items, 57
  -dependent decarboxylation, 69
  depletion, first-order reaction, 33
  extracelluar, 35
  -free items, 57
  hygiene assays, problems, 45
  intracellular, 36–37
  in living cell, 57
  in mammalian cells, 35
  measuring, 32
Adenylate kinase (AK), 35, 50, 51
  reaction, 47, 48
Aequorea victoria (jelly fish), 88, 174*t*
Aequorin-based bioreporters, 88–94
  Anabaena sp. PCC 7120, 97
  functional recombinant aequorin, 88
  sensitivity to Ca^{2+} changes, 89
  use in *E. coli*, 97
Albendazole, 100
Alcohol deshydrogenase (*ADH1*) gene, 87
Alicagenes eutrophus 2050, 86
Alicagene eutrophus BR6020 (pUTK2), 86, 94*t*
Alcaligenes eutrophus CH34, 83
Aliivibrio fischeri, 6, 10, 15, 70, 141, 191
Alteromonas, 15
American Society for Testing and Materials (ASTM), 68, 70, 73

G. Thouand and R. Marks (eds.), *Bioluminescence: Fundamentals and Applications in Biotechnology–Volume 2*, Advances in Biochemical Engineering/Biotechnology 145, DOI: 10.1007/978-3-662-43619-6,

Ames reverse mutation assay, 139
Ames test, 83
Aminoluciferin, 40
Anabaena CPB4337, 80, 86, 94*t*, 102, 112
Anabaena sp. PCC7120, 88, 94*t*, 95*t*, 97
Androgens, 105
Anthracycline daunorubicin (DNR), 194
Anthracycline idarubicin, 197
Anthracyclins, 197, 210, 211
Antibiotic metabolites, 157
Antibiotic residues, 155, 157
  in food, detection methods, 159
    biosensors, 167–179, *see also* Biosensors
    confirmatory analysis of, 159–161
    screening methods, 161, 164, 165–166*t*, 167
  maximum residue limits (MRLs), 158, 159
  multiresidue methods, 160
Antibiotic resistance, 139, 154, 155, 156, 179
  of *Salmonella* strains, 157
Antibiotic use
  and associated risks, 155–157
    food, 157
  control of, 157–159
  in the EU, 156*t*
  nontherapeutic antibiotics, 157
  in Switzerland, 156*t*
Antibody, 169*t*, 204
  polyclonal antibody, 169*t*
  monoclonal antibody, 169*t*
  and receptors, 169*t*
Apoaequorins, 74, 88, 89, 96*t*, 97
Apoptosis, 193, 197, 203
Arabidopsis thaliana, 6, 20
Ara-CMP, 196, 198
Ara-CTP, 196, 197, 198, 199, 200, 204, 206, 207, 208*f*, 209*f*
  accumulation in leukaemic cells, 203*f*
  on biosensor, 201*f*
    assay procedure, 202*f*
    treated with, 205*f*
  validation of measurement, 203
Ara-uracil (Ara-U), 196, 198, 199
Armillaria, 72, 77
Asilomar approach, 23
Autotrophic dinoflagellates, 73
Avalanche photodiodes, 8

B

Bacillus subtilis, 80–81, 88, 173, 175*t*, 177*t*, 178
Bacillus subtilis BGA, 165*t*, 166*t*, 167
Bacillus subtilis BR151 (pBL1/p602/22lux), 80, 93*t*
Bacitracin, 176*t*, 177*t*
Bacterial ATP, 35, 45
  assay, 36*f*
Bacterial bioluminescence, 15–17, 68, 72
Bacterial biosensors, 209, 210, 211, *see also*Bioluminescent bacterial biosensors
  assays
    assay procedure, 202*f*
    Box–Whisker plot of, 208*f*
    clinical testing, 207
  bioluminescent biosensor development, 199–202
    effect of ara-CTP on, 200*f*, 201*f*
  for chemotherapy sensitivity testing
    cytarabine sensitive *E. coli* host, 199
    mode of action of target drug, 198
Bacteriuria detection, 33
Benzene, toluene, ethylbenzene and xylene (BETEX), 80, 82, 91*t*, 92*t*
BioBrick, 8
Biolight, 6
Bio-lighting
  concerns, 24
  feasibility, 9
    competing with conventional lighting, 10–11
    energy output, 9–10
    photosynthetic efficiency, 9
Bioluminescence, 4, 68
  application, 5
    aesthetics, 6–7
    communication with humans, 7–8
    communication with other engineered systems, 8
    illumination, 5–6
  versus LEDs, 7
  parts and modules in nature, 12
    bacterial bioluminescence, 15–17
    firefly luminescence, 12–15
    natural luminescence systems, 17–18
  in response to genotoxicants, 145*f*
  and stimulation, 101
Bioluminescent bacteria, 69
  on effect of pollutants on eukaryotic organisms, 72
  Gram-positive versus -negative bacteria, 81–82
Bioluminescent bacterial biosensors
  for drug sensitivity, 191
    bioluminescent reporter technology, 191

intracellular antimicrobial effects, 192–193
real-time monitoring of antimicrobial effects, 192
future perspectives, 209
additional chemotherapy biosensors, 211
applications with other drugs, 211
cytarabine biosensor, 210
multi drug testing, 210
rapid testing in other cancers, 211
Bioluminescent fungi, 72
metabolism of, 72
nonenzymatic mechanism for, 73
Bioluminescent microorganisms
for bioremediation programs, 84–86
in ecotoxicity testing of pollutants, 69*f*
in environmental monitoring, 68–74
main features and applications of, 75
for wastewater toxicity evaluation, 84–86
Bioluminescent organisms
to assess mixture toxicity, 103
whole toxicity assessment, 103–105
hormetic response of, 101–103
mixture toxicity, commonly used models, 107–108*t*
and toxicology of mixtures, 105–114
Bioluminescent trees, 4, 6, 23*f*, 26
Biophobes, 99
Bioreporters
duplicating different elements, 140
Pseudomonas putida-based Cd/Zn/Pb-bioreporters, 139
Biosensor assays, 167, 168, 178, 207
procedure, 202, *see also* under Whole-cell biosensors
reproducibility and stability, 204–206
assay precision using QC samples, 206*t*
sensitivity and specificity, 204
validation of, 202–203
ara-CTP measurement, 203–204
Biosensors
assay multiplexing, 171
definition, 167
for detecting antibiotics in food, 167–168, 169–170*t*
portable devices for field use, 171
principle of, 168*f*
proteins in, 171
in synthetic biology, 22
whole-cell biosensor, *see* Whole-cell biosensors
Bliss additivity, 106
Bovine serum albumin (BSA), 39, 45, 46, 52
Burkholderia sp. RASC c2, 79, 85, 94*t*

C
Cadmium (Cd), 76, 77, 81, 85, 90*t*, 92*t*, 93*t*, 101, 109, 146
Pseudomonas putida-based Cd-bioreporters, 139, 146
-responsive reporter, 147*f*
cadR (Cd-responsive regulator gene) promoter, 146, 147*f*
Calcium (Ca), 37, 88
cytoplasmic free calcium, 89
effects of rhamnolipids on, 81
eukaryotic biosensor using, 89
intracellular calcium dynamics, 88, 89
toxicants disruption on, 89
triggered calcium signature, 97
DD-carboxypeptidase, 169*t*
Caspases assay, 40
Cell arrays, 83
Ceratocorys horrida, 73
Charge-coupled device (CCD) camera, 83
Chassis organisms selection, 18–22
E. coli, 18
luminescent bacteria from household waste, 18, 19*f*
tobacco plant luminograph, 14*f*, 18
Chemical mutagens, 102
Chemical predictors, 98
Chlorinated aliphatic hydrocarbons (CAHs), 79
Chlorophenols, 77, 78, 82, 85, 91*t*, 93*t*, 94*t*
Chlorophyll a, 35
Codex Alimentarius, 158
Combination index (CI)-isobologram equation, 109
Concentration addition (CA) model, 106
Confirmatory methods, 159–160
for veterinary drugs, 161*t*
Confirmatory test, 159
Copper (Cu), 76, 77, 80, 81, 84, 85, 86, 87, 90*t*, 91*t*, 92*t*, 93*t*, 94*t*, 95*t*, 101, 109, 110, 111
Coupled enzyme assays, 47
kinetic assays of, 48–49
of ATP-depleting enzymes, 51–52
of ATP-forming enzymes, 49–51
metabolites, endpoint assays of, 47–48
D-cycloserine, 176*t*, 177*t*

Cymnocyprus przewalskii, 72
CYP450 assay, 40
D-cysteine, 14
Cytarabine (Ara-C), 198
  in acute myeloid leukaemia, 195–196
    future of, 196*f*
  apoptosis, 197, 199
  biosensor response, 200*f*
  comparison of results, 208*f*, 209*f*
  drug inactivation, 198
  efficacy, 197
  high-dose, 196–197
  resistance to, 199
  S phase activity, 199
Cytarabine biosensor, 210
Cytidine deaminase (cdd), 197, 198
  -deficient mutant, 199
Cytidine triphosphate (CTP), 47
Cytochrome c, 196
Cytotoxicity test (Promega CellTiterGlo®), 209

D
Daunorubicin (DNR), 194, 197, 210, 211
Deoxycytidine kinase (dCK), 196, 197, 198, 199, 202, 204, 210
  IPTG induction of, 200*f*
  IPTG-induced expression of, 205*f*
Dinaphthofurans, 105
Dino Pet (Yonder Biology), 6
Dinoflagellates, 73
  bioluminescence, 17, 73
  toxicity bioassay, 73
    of heavy metals, 73
Directed evolution process, 27, 141, 142
Disease control, 155, 156
DmpR (Dimethyl phenol regulatory protein), 142, 143
DNA, 196, 197
  barcodes, 24
  -based aptamer, 171
  cDNA, 97, 199, 211
    of ribosome binding sites (RBSs), 145
    and synthesis, cost of, 13*f*
  -damaging agents, 144, 146, 147, 175*t*, 176*t*
  natural DNA, 26
  recombinant technology, 23
  sequencing, 24
  shuffling' procedure, 142
  synthetic DNA, 25
  watermarks, 24
Dodecyltrimethylammonium bromide (DTAB), 37
Dose-addition model, 106
Douglas fir (*Pseudotsuga menziesii*), 21
Doxorubicin, 197, 210, 211
Dual-reporter construct, 147

E
Effect-based identification of key toxicants, 105
Elatiridae(click beetles), 68
Energy charge (EC), 37
Environment Canada, 68
Environmental biosentinels, 7
Environmental monitoring
  bioluminescent microorganisms in, 68–74, *see also* Bioluminescent microorganisms
  E. coli HB101 in, 76
Enzyme–linked immunosorbent assays (ELISA), 160, 164
EquRay, 112
Escherichia coli, 74, 18
  -based biosensors, 173
  cytarabine sensitive *E. coli* host, 199
  E. coli HA1, 200
  E. coli HB101 (pUCD607), 76, 79, 110
    acute toxicity assays, 76
    in ecotoxiciy of herbicides, 79
    in heavy metal toxicity, 77
    online biosensing, 79
    for toxicity of Zn, Cu, and Cd mixtures, 77
    Zn sensitivity, 76, 80
  E. coli MG1655, 199
  E. coli RFM433 GC2, 82, 83
  environmental monitoring, 76
Ethylenediaminetetraacetic acid (EDTA), 36–37, 77
Eukaryotic bioreporters, 87–88
  prokaryotic to eukaryotic genes, 87–88
European Commission Decision 2002/657/EC, 159, 160, 161, 164, 179
European Union (EU) legislation, 155
Extracellular ATP, 35, 36*f*

F
FB12 luminometers (Berthold Detection Systems), 35
Firefly (*Photinus pyralis*), 69
  luciferase

autoluminograph of transgenic tobacco plant, 14*f*
expressed in *E. coli*, 16*f*
luminescence, 12–15
Firefly luciferase reaction
in analytical purposes, reasons for, 33
calibration of assays, 52–56, 53*f*
optimization of reagents, 44
assays of ATP, 44–46
assays of luciferase, 46
coupled enzyme assays, 47
sample pretreatment, 34
bacterial concentration, 34
dilution, 34–35
to remove specific pools of ATP, 35–36
resuscitation, 36
stabilization of reagents, 52
First International Symposium on Bioluminescence and Chemiluminescence, 33
Flavin mononucleotide ($FMNH_2$), 15, 19, 138, 191
Fludarabine, 197, 200, 204
Fluorescence, 8, 57, 146, 161, 164
Fluoroimmunoassays, 160
Fluoroluciferin, 40, 46
Fluoroquinolones, 168, 171, 175*t*
Food safety, 158
Foxfire, *see* Fungal bioluminescence
Fragment-based QSARs, 98–99
Free-ion metal and toxicity, 77
Freshwater bioreporters, 86–87
Anabaena CPB4337, 86, 87
Janthinobacterium lividum YH9-RC, 86
frp (*flavin oxidoreductase*) gene, 88
Functional recombinant aequorin, 88
Fungal bioluminescence, 17, 73
Panellus stipticus, 18*f*

G

β-galactosidase-expressing biosensor, 178
Gas chromatography, 67, 161
Genetic containment mechanisms, 25*f*
Genetic function approximation, 99
Genetic manipulations
additional manipulations, 144–147
of host strain and expression system, 139–140
of *lux* reporting elements, 143–144
of sensing element, 140–143
random mutagenesis, 142
and synthetic biology, advent of, 144–147
Genetically engineered microbial bioreporters, 138
Genetically modified organism (GMO), 4, 12, 23, 24, 26
gfp (*green fluorescent protein*) gene, 138, 145, 146, 147*f*, 174*t*, 175*t*, 176*t*
GFP (green fluorescent protein), 7, 8, 147, 173
Gliceraldehyde 3-phosphate deshydrogenase (*GPD*) gene, 87
Glofish, 7
Glycerol assay, 47
Glycerol kinase (GK) reaction, 48
Glycopeptides, 176*t*
Gram negative bacteria, 69, 81–82
Alcaligenes eutrophus CH34, 83
E. coli, *see Escherichia coli*
P. fluorescens, *see Pseudomonas fluorescens*
Gram-positive bacteria, 81–82
B. subtilis, *see Bacillus subtilis*
S. aureus, *see Staphylococcus aureus*
Streptomyces lividans, 82
Granulocyte-macrophage colony stimulating factor (GM-CSF), 197
Growth promotion, 155, 156*t*, 157
grpE genes, 139, 140, 175*t*

H

Highest occupied molecular orbital (HOMO), 98, 99
ecological risk of (EHOMO), 100
High-performance liquid chromatography (HPLC) methods, 160, 203
Hormesis, 101
bioluminescent microorganisms and QSAR models, 97–100
mechanism of toxic action (MOA), 98
response of *V. fischeri*, 101
Human equilibrative nucleoside transporter (hENT1), 196, 197
Humic acids (HA), 111
Hydrophobic pentachlorophenol, 139
Hydroxilamine oxidoreductase (*hao*) promoter, 84

I

Idarubicin, 197, 210, 211
Identification points (IP), 160
Imidazolium groups, 100
Immunoanalytical methods, 160, 161
Inducible bacterial biosensors, 173

Inducible bacterial biosensors (*cont.*)
algorithm, 178, 179
antibiotic-inducible effect-specific whole-cell biosensors, 179
compound-specific, 176–177*t*, 178
effect-specific, 175–176*t*, 178
E. coli-based biosensors, 173
Inductively coupled plasma (ICP), 67
-mass spectrometry (ICP-MS), 80
Integrated Fuzzy Concentration Addition-Independent Action Model (IN-FCIM), 111
International Organization for Standardization (ISO), 68
Intracellular ara-CTP (Intracellular cytosine arabinoside triphosphate), 204
Intracellular ATP, 33
extraction, 36–37
Intracellular messengers, 88
Isopropyl-1-thio-*β*-D-galactopyranoside (IPTG), 146
-controlled *ars*R gene, 145
E. coli HA1 in, 200*f*
inducible dCK expression, 199, 204*f*, 205*f*
-inducible promoter, 199, 210
Ivermectin, 100

J

Japanese firefly (*Luciola cruciata*) luciferase, 15
Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, UK, 157

K

Kinetic assay, of metabolites, 59
of ATP-depleting enzymes, 51–52, 53*f*
of ATP-forming enzymes, 49–51, 53*f*
of glycerol, 48–49
urea, 49
Kinetics, of luciferase reaction, *see* Luciferase reaction kinetics

L

β-lactamase, 169*t*
β-lactams, 176*t*, 177*t*, 178–179
lacZ, 138
Lampyridae (fireflies), 68
LB-agar matrix, 83
Leukaemia, clinical need, 193–198
accumulation of ara-CTP in, 203*f*
adult patients
survival of, 195*f*
treatment-related mortality, 196
ara-C, 195, 196, *see also*Cytarabine (Ara-C)
chemotherapeutics in, 194
drug response profile, 197
targeted chemotherapy, 197
FLT3/ITD and NPM1 mutant status in, 194*f*
internal tandem duplication (ITD), 193
Leukaemic cell death, 199, *see also*Apoptosis
Light-dependent resistors, 8
Light-emitting
diodes (LEDs), 5
reagents, 47, 53, 54
organisms, 8, 15, 69
reactions, 8, 27
Light-sensitive proteins, 8
Lights-off
bioassays, 81
bioreporters, 74, 80, 83, 84, 85, 86
recombinant microorganisms, 82
Linear alkylbenzene sulfonate surfactant (LAS), 84, 93*t*
Lingulodinium polyedrum, 73
Liquid chromatography (LC)-mass spectrometry (MS), 67, 160
Location parameter, 112
Loewe additivity, 106
Lowest unoccupied molecular orbital (LUMO), 98, 99
ecological risk of (ELUMO), 100
luc gene, 69
luc-based bioreporters, 74
Luciferase, 8, 72
assays, optimization of reagents, 46
in controlling luminescence, 22–23
measuring, 32
light parameters, 58–59
luciferase inhibitors, 59
read-outs, 58–59
selection, 37–38
ATP assays, 38–39
reporter gene assays, 38
Luciferase reaction kinetics, 41–44
double logarithmic standard curves, 42*f*
intensity and decay of light emission, 42*f*
linearity and detection limit, 43*f*
luciferase-oxyluciferin—AMP complex, 43
peak height and decay rate, 41*f*
rules, 41
Luciferin

analogues and derivatives, 40
D-luciferin, 14, 27, 38, 39, 40, 41, 46, 52, 87
measuring, 32
oxidative decarboxylation of, 33
Luciferin-regenerating enzyme (LRE), 14
LRE cycles, 16*f*
Luciola cruciata, 44, 46
Luminescence, 26, 70
biosensing applications, 28
communication applications, 28
improving and altering, 26–27
alterations, 27
microenvironments in, 27
optimization, 26–27
Luminosity functions, 10
photopic, 10, 11*f*
scotopic, 10, 11*f*
from typical streetlamps, 11*t*
LUMISTox
applications, 75*t*
from Beckman Instruments, 70
LUMITOX®, 73
applications, 75*t*
lux bioreporters, 74, 138
reporting elements, 143–144
lux operon, 15, 16, 20, 26, 80, 81, 83
lux genes, 76, 84, 103, 139, 174*t*, 191, 192, 201, 210
luxA, 15, 87, 144
luxAB, 69, 79, 80, 82, 84, 86, 92*t*, 93*t*, 94*t*, 142, 143, 145*f*, 177*t*
oxidation of a reduced flavin mononucleotide ($FMNH_2$), 138
luxB, 15, 87, 144
luxC, 15, 69, 87, 138
luxCDABE, 76, 80, 82, 85, 88, 90*t*, 91*t*, 92*t*, 93*t*, 94*t*, 95*t*, 138, 139, 140, 143, 144, 145*f*, 175*t*, 176*t*, 177*t*, 191, 199, 210
luxCDABEG, 15, 20
luxD, 15, 69, 87, 138
luxE, 15, 69, 87, 138
Lyophilized biosensor, 205
testing, 206*f*

M

Macrolide/streptogramin/lincosamide (MLS) antibiotics, 160
Macrolides, 177*t*
Mammalian ATP, 35
Mercury (Hg), 81, 90*t*, 101
Metabolic bioreporters, 74
Meticillin-resistant *Staphylococcus aureus*, 155
Mg-ATP, 45
Michaelis-Menten equation, 32, 41
Microbial cell, 170*t*
Microbial engineering
engineered gene circuit diagrams, 147*f*
protein expression, 145
Microbial growth inhibition tests, 155
Microbial Home project (Philips), 6
Microtox™, 70, 71, 74, 109
applications, 75*t*
disadvantage, 71–72
limitation, 74
MicrotoxOmni™, 70
Mitomycin C (MMC), 139
Mixture toxicity, 69*f*
use of bioluminescent microorganisms in, 103, 105–106, 109–114
whole-toxicity assessment, 103–105
Models for Assessing and Forecasting the Impact of Environmental Key Pollutants on Freshwaters and Marine Ecosystems and Biodiversity (MODELKEY), 105
Modulators, 99
Morantel, 100
Multicolor luciferase assays, 69
Multiresistant strains, 155
Mycenoid, 72

N

N-acetylcystein, 50
NADPH-reductace, 72, 88, 201
National Center for Biotechnology Information Genbank database, 12
sequence information deposited in, 13*f*
Natural estrogens, 105
Naturally bioluminescent microorganisms, in environmental monitoring, 68–74
Needless prescriptions for viral infections, 155
Nickel (Ni), 76, 77, 85, 93*t*, 109
Nicotiana tabacum
autoluminescent seedlings, 21*f*
plastids, 20
Nitrosomonas europaea ATCC 19781 (pHLUX20), 84, 93*t*
Non-Hodgkin's lymphoma, 197
Non-polar narcosis mode of action, 98
Norwegian spruce (*Picea abies*), 21
N-phenyl-2-naphthylamine, 105
Nucleoside analogue drugs, 211

O
Omphalotus, 72
Open reading frame (ORF), 19, 140
Ophiopsilla californica, 71
Ophistoproctus soleatus, 71
Organization for Economic Cooperation and Development, 68
Organotins, 78, 91*t*
Orthogonality, 25–26
  genetic code orthogonality, 25
  refactoring cell's codon usage, 25
Over-the-counter drug, 155
Oxygen, 32, 34, 39, 52, 69, 73, 88, 138, 172, 174*t*, 191, 197
Oxyluciferin, 14, 32, 43, 44

P
pDNlux plasmids, 80, 90*t*, 92*t*
Pentachlorophenol (PCP), 76, 78, 79, 80, 91*t*, 93*t*, 139
Phangodidae (railroad worms), 68
Phenol contamination, 81, 82, 85, 86, 94*t*, 101, 143, 145, 192
Photinus pyralis, 12, 37, 46
Photobacterium, 15, 69
Photobacterium leiognathi lux operon, 16*f*, 20
  strain TANI-1, 71
Photobacterium phosphoreum, 70, 71, 75*t*, 100, 102, 113
Photodiodes, 8
Photomultipliers, 8
Photorhabdus, 15, 69
Photorhabdus luminescens, 143, 191
  luxCDABE operon of, 144
Photosynthetic efficiency, 9
Photosynthetically active radiation (PAR), 9
Pimpephales promelas (fathead minnow), 85
Plankton, 4, 6
Plasmids, 25, 26, 81, 89, 145
  multicopy plasmid-based fusion, 140
  pBBR1MCS-2, 199, 210
  pDNlux, 80
  pESK004, 82
  pLITE2, 82
  pSLlux, 80
  pTrcHUMdCK, 199, 210
  pUCD607, 76, 79, 80
  pUTK2, 85, 86
  recA::lux plasmid comparison, 139
  *sulA::lux*CDABE fusion, 141
  TOL, 142
Plastid translation machinery, 19
Pollutants, 138
  evaluation of ecotoxicity of, 67–115, *see also*Aequorin-based bioreporters; Naturally bioluminescent microorganisms, in environmental monitoring; Recombinant bioluminescent microorganisms
Populus trichocarpa, 20
Predictor variables, 97
Proteases assay, 40
Pseudomonas
  luxCDABE cassette, 143
    assumptions, 143–144
  naphthalene degradation pathway in, 142
  naphthalene detection, 138
  phenol bioreporter, 143
Pseudomonas fluorescens, 81, 92*t*
  P. fluorescens 10586 (pUCD607), 76, 77, 78, 79, 80, 91t, 110
  P. fluorescens 8866 Tn5luxCDABE, 91*t*
  P. fluorescens DF57-40E7, 84, 92*t*
  P. fluorescens OS8 (pDNlux), 80, 92*t*
  P. fluorescens OS8::Knlux, 80, 92*t*
  P. fluorescens Shk1, 92*t*
Pseudomonas luminescens luciferase, 80
  luxCDABE, 82, 86
  toxicity lights-off bioassays, 81
Pseudomonas putida
  P. putida-based Cd/Zn/Pb-bioreporters, 139
  P. putida F1, 76
  P. putida mt-2, 97
Pseudomonas Shk1, 98
pSLlux plasmids, 80, 90*t*
pUTK2 plasmid, 85, 86, 94*t*
Pyridinium groups, 100
Pyrocystis noctaluca, 73
Pyrrolidinium groups, 100
Pyruvate kinase (PK) reaction, 47

Q
Quantitative structure-activity relationship models (QSAR models), 97, 105, 111, 114
  -based models, 109
  bioluminescent microorganisms and, 97–100
QwikLite®, 73
  applications, 75*t*
  test, 73

R
Radioimmunoassays, 160

Rapid testing to cancer, 198, 211
Recombinant bioluminescent microorganisms, 74–83
aequorin-based bioreporters, 88–94
bioreporters specially designed, 84–86
eukaryotic bioreporters, 87–88
features and applications of
A. awamori 66A, 96*t*
A. eutrophus BR6020 (PUTK2), 94*t*
A. niger, 96*t*
Acinetobacter sp. DF4/pUTK2, 94*t*
Anabaena sp. PCC 7120 (pBG2001a), 95*t*
Anabaena sp. PCC7120 CPB 4337, 94*t*
B. subtilis BR151(pBL1/p602/22lux), 93*t*
B. subtilis BR151(pCSS962/pBL1), 93*t*
Burkholderia sp. RASC c2, 94*t*
E. coli AB1 157 (pMMB66EHAEQ), 95*t*
E. coli GC2, 91*t*
E. coli HB101 (pUCD607), 90*t*
E. coli MC1061 (pDNlux), 90*t*
E. coli MC1061 (pSLlux), 90*t*
J. lividum YH09-RC, 94*t*
N. crassa, 96*t*
N. europaea ATCC 19781 (pHLUX20), 93*t*
P. fluorescens 10586 (pUCD607), 91*t*
P. fluorescens 10586/FAC510, 91*t*
P. fluorescens 8866 Tn5luxCDABE, 91*t*
P. fluorescens DF5740E7, 92*t*
P. fluorescens OS8 (pDNlux), 92*t*
P. fluorescens OS8::Knlux, 92*t*
P. fluorescens Shk1, 92*t*
P. putida F1 (pUCD607), 92*t*
P. putida F1 Tn5luxCDABE, 92*t*
Pseudomonas spp. PM6, 92*t*
R. leguminosarum bv *trifolii* (pUCD607), 93*t*
R. leguminosarum bv *trifolii* TA1-Tn5luxAB, 93*t*
S. aureus RN4220 (p602/22lux), 93*t*
S. aureus RN4220 pTOO02, 93*t*
S. cerevisiae BY4742 (pEVP11-Aeq89), 95*t*, 96*t*
S. cerevisiae H208-3B (pGAPAQ), 96*t*
S. cerevisiae H208-3B (pGAPAQ1), 95*t*
S. cerevisiae JK9-3da (pEVP11/AEQ), 95*t*
S. cerivisiae BLYEV, 95*t*
S. cerivisiae W303-1B LucD, 95*t*
S. typhimurium TA104 pr1, 91*t*
Stenotrophomonas 664 (pUTK2), 94*t*
Streptomyces lividans (pESK00), 93*t*
Synechocystis sp PCC6803, 95*t*
freshwater bioreporters, 86–87
specific soil bioreporters, 84
Recombinant DNA technology, 23
Reduced flavin mononucleotide ($FMNH_2$), 138, 191
Relative light units (rlu), 52
Reporter gene assays, 38
Repressor protein, 170*t*
Resistant pathogens, 154
Response variables, 97
RFP (red fluorescent protein), 147
Rhamnolipids based-biosurfactants, 81
Rhizobium leguminosarum biovar *trifolii*, 76
Riboflavin, 18
Rose (*Rosa hybrida*), 21

S
S phase activity, 199
Saccharomyces cerevisiae, 87
S. cerevisiae H208-3B (pGAPAQ1), 95*t*, 96*t*
S. cerevisiae hER, 87
S. cerevisiae W303-1B, 87
S. cerevisiae W303a, 87
S.cerevisiae BY4742 (pEVP11-Aeq89), 95*t*, 96*t*
S.cerevisiae JK9-3da (pEVP11/AEQ), 95*t*
Salmonella typhimurium, 83, 139
TApr1, 83
SBP (sulfonamide binding protein), 170*t*
Screening methods, 159, 160
for antibiotic residues, 161, 164
biosensors as, 168, 179
detection capability, 161
microbial growth inhibition, 165–166*t*
growth inhibition tests, 155
whole-cell biosensors, *see* Whole-cell biosensors
Screening test, 159
Self-medication, 155
Semi-coke leachates, 81
Shewanella, 69
Shk1 (Shock 1) lights-off bioreporter, 85
Single-ended reporters, 147
ssDNA aptamer, 168, 170*t*
Staphylococcus aureus, 80–81, 176*t*
meticillin-resistant, 156
S. aureus RN44220 (p602/22lux), 80, 81, 93*t*

S. aureus RN44220 (pTOO02), 93*t*
Stenotrophonomas 3664, 86
Stenotrophonomas 664 (pUTK2), 86
Substituted phenols, 105
Sugar cane (*Saccharum* sp.), 9
sulA, 140
Swann Report, 158
Synthetic biology
  aims, 4
  controlling luminescence, 22–23
    biosensors, 22
  modularization, 5
Synthetic estrogens, 105

T
TANI-1 (ToxScreen I version), 71
Tetracyclines (TC), 160, 176*t*
Tetrahymena pyriformis (ciliate), 85
Tox-Alert®, 70
  applications, 75*t*
Toxic equivalency factor (TEQ)-based methods, 109
Toxicity bioassay, 67, 73
Toxicity fingerprinting, 79
Toxicophores, 99
ToxScreen, 71
  applications, 75*t*
Transgenic bioluminescence, 14*f*, 17*f*
Tributyltin (TBT), 78
Trichloroacetic acid (TCA), 37
Trichlorophenol (TCP), 78, 94*t*, 101
Triphenyltin (TPT), 78

U
Ultraviolet (UV) light, 7, 102
U.S. Environmental Protection Agency (US EPA), 68
Urea assay, 49

V
Vancomycin, 177*t*
Vancomycin-resistant enterococci, 156
Veterinary antibiotic use, 155, 156, 156*t*, 157, 158, 160, 164, 167, 179
Vibrio, 69
Vibrio fischeri, 15, 70, 72
  luxCDABE genes, 85
Vibrio harveyi, 15, 82, 191
Vibrio logei, 71
Vibrio qinghaiensis sp.-Q67, 72, 75*t*
Viral internal ribosome entry site (Viral IRES), 16
VITOTOX® test, 83, 91*t*, 141

W
Weak acid respiratory uncouplers (WARUs), 98, 99
Weibull equation, 102
Whole-cell biosensors, 155
  assays, 171, 172, 178
  compound-specific sensors, 171, 172
  disadvantages to, 172–173
    choice of reporter gene, 173
  effect-specific sensors, 171
  inducible bacterial,*see also* Inducible bacterial biosensors, 172*f*
  reporter genes and proteins commonly used in, 174
Whole-cell-based bioassays, 138
Wild-type luciferase, 39, 52
World Health Day 2011, 158
World Health Organization (WHO), 158
World Organization for Animal Health, 158
World Veterinary Association, 158

X
Xenorhabdus, *see Photorhabdus*
xylR regulatory gene, 142
XylR regulatory protein, 97, 142

Y
ybiJ::*gfpmut2* fusion, 145
Yeast internal ribosome entry site (Yeast IRES), 87
yqiF gene promoter, 142
yqjF::*luxCDABE* fusion, 142

Z
Zinc (Zn), 76, 77, 80, 81, 85, 86, 89, 90*t*, 91*t*, 92*t*, 93*t*, 94*t*, 95*t*, 96*t*, 101, 110, 139