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Gérald Thouand
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Bioluminescence: Fundamentals and Applications in Biotechnology—Volume 1

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Bioluminescence: Fundamentals and Applications in Biotechnology–Volume 1

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Part I
Fundamentals of Bioluminescence

Eco-Evo Bioluminescence on Land and in the Sea

Yuichi Oba and Darrin T. Schultz

Abstract This review discusses the evolution of bioluminescence organisms that inhabit various environments based on the current understanding of their unique ecologies and biochemistries. As shown here, however, there are still many unanswered questions regarding the functions and mechanisms of bioluminescence, which should be investigated in further studies. To facilitate future research in this field, we introduce our recent attempt, the bioluminescent organism DNA barcode initiative. This genetic reference library will provide resources for other scientists to efficiently identify unstudied bioluminescent organisms, focus their biochemical and genetic research goals, and will generally promote bioluminescence as a field of scientific study.

Keywords Aposematism · Coelenterazine · Counter-illumination · Cypridinid luciferin · DNA barcoding · Ecology · Evolution · Symbiotic luminescence

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1 Introduction

Bioluminescent organisms occur in a wide variety of habitats, from tropical jungles to barren fields, from sunny beaches to dark caves, and from oceanic surface waters to abyssal depths. Those species that are aquatic are largely confined to the oceans whereas only a few occur in freshwater. Bioluminescent taxa have been reported from bacteria to vertebrates, in fact from most branches on the tree of life. However, some branches are species-rich, such as teleosts, crustaceans, cnidarians, and coleopterans, whereas others contain few or, like plants, tetrapods, arachnids, and lepidopterans, have no known bioluminescent representatives (Fig. 1). The biological and ecological functions of bioluminescence vary from species to species, but it seems likely that counter-illumination is predominant in marine species, and aposematism is abundant in terrestrial species. Coelenterazine, a substance involved in the luminescence reaction, is commonly used by disparate marine taxa, whereas firefly luciferin is used only in the terrestrial elateroid beetles. In this chapter, we review the ecology and evolution of luminous organisms, and discuss the possible reasons why some functions, habitats, chemical substrates, and taxa are preferred or avoided in the world of bioluminescence.

2 Function of Bioluminescence

2.1 Self-defense

Many different biological and ecological functions have been proposed for bioluminescence [1, 2], but the most parsimonious explanation is that it is used as an antipredator defensive device. Here we categorize the various defensive reactions by luminous organisms aimed at predators into three types: startling (or deterring), misdirection (or distraction/obstruction of sight), and aposematism (warning display).

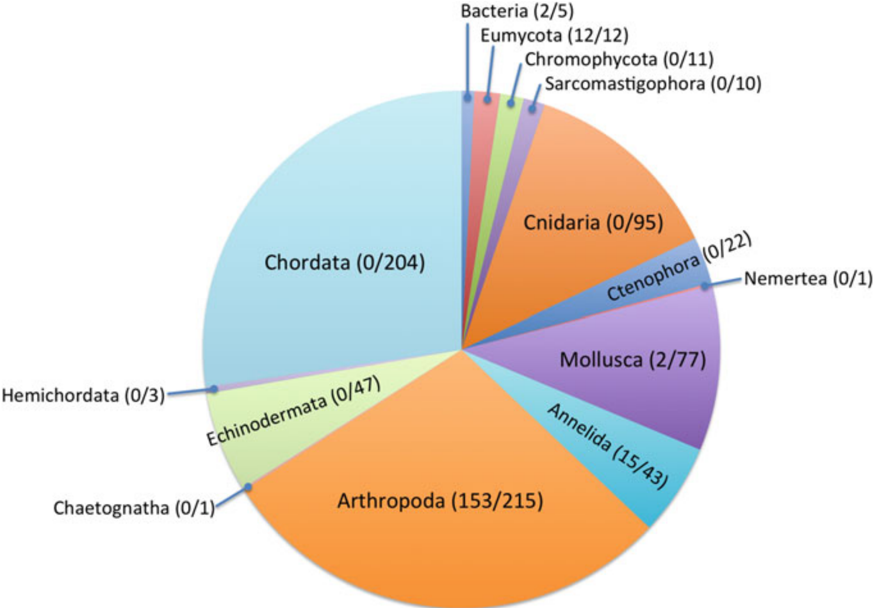

Fig. 1 The diversity of bioluminescent organisms as proportions of the named 746 genera. The fraction indicates the number of terrestrial genera relative to the total number of genera. Numbers are based on Herring [1] and the supplemental material in Haddock et al. [2]. The genera not certain to be luminous in Herring [1], and "doubtfully" luminous genera of sponge and bryzoan [2] are excluded. Numbers of genera in some groups are modified according to recent publications: Bacteria [139], Lampyridae [173], Phengodidae [8], Rhagophthalmidae [174], Gastropoda [109], Oligochaeta [102], Homalisidae (Omalisidae) [81], and Fungi [97]. Nematode *Heterorhabditis* is excluded because symbiotic luminous bacteria *Photorhabdus* does not emit visible light in the nematode [100]. For compatibility with the list by Herring [1], the classification of Division and Phylum is followed in the systematics by Parker [95]

Sudden flashes in dark surroundings have been shown to startle, deter, and stun or temporarily blind predators regardless of the prey's palatability. For example, the mesopelagic squid, *Taningia danae,* uses its arm-tip photophores to flash in bursts while attacking bait rigs [3]. Moreover, in the firefly squid *Watasenia scintillans* (Fig. 2), the nervous-system controlled arm-tip photophores are thought to flash in bursts when attacked by predators [4, 5]. This seems to be similar to cases in which the eye-spots on the surfaces of the hind wings of some Lepidoptera startle predators by their sudden exposure [6], an example of Batesian mimicry in which reflective owl eyes are the model. However, startling bioluminescence is probably not Batesian mimicry, as it does not imitate any predator models. The exceptions include some phengodid beetle larviform female adults, and the adults of click beetles. The female phengodids *Diplocladon* and *Rhagophthalmus* curl their bodies around their eggs to protect the brood, and when disturbed emit light from small circular photophores lining the body (Fig. 3).

Fig. 2 Firefly squid *Watasenia scintillans* collected in Toyama Bay, Japan. This picture shows the interspersed photophores on the ventral side of the mantle that face down as the creature swims. The larger light organ on the tip of arms is assumed to be used for stunning predators. Photo courtesy of So Yamashita

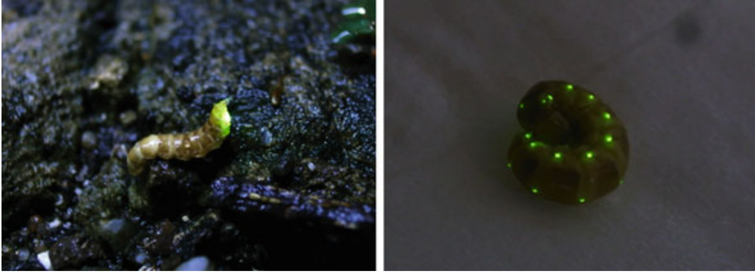

Fig. 3 Luminescence of the phengodid beetle *Rhagophthalmus ohbai* female adult at Ishigaki Island, Japan. Attracting a male (*left*) and after copulation (*right*)

Ohba [7] hypothesized that the light emission may function to startle predators by mimicking the shining eyes of nocturnal birds. The adults of the luminous click beetles (e.g., Jamaican *Pyrophorus* species) possess a pair of oval photophores at the outward-angled posterior margins of the prothorax [8], and the luminescence looks like the shining eyes of nocturnal animals found in forests at night (VB Meyer-Rochow, personal communication). Thus, the luminescence of phengodid and click beetles may be an example of a startling function evolved in the presence of a sympatric predator model.

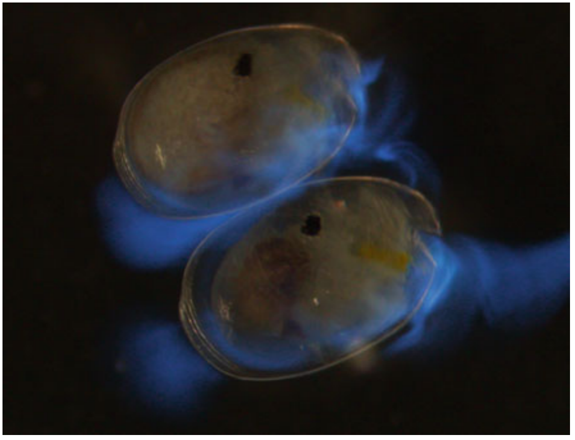

Fig. 4 Coastal ostracod *Vargula hilgendorfii* collected at Seto Inland Sea, Japan. This blue luminous cloud was discharged upon electro-stimulation. Photo courtesy of Ken-ichi Onodera

Luminescent secretion clouds discharged by agitated bioluminescent organisms in aphotic or very dim seawater are assumed to cause predators to lose sight of the prey or become distracted while the creature escapes. This is similar to the squid, octopus, and other cephalopod behavior of releasing a "smokescreen" of dark ink to obscure its position from predators when disturbed. Some luminous squid, shrimp, and ostracods (Fig. 4) discharge luminous clouds into the seawater, primarily to act as a smoke screen against predators while making an escape [2]. Some luminous species discharge luminous droplets or mucus as decoys and flee when attacked, for example, terrestrial centipedes [9], freshwater limpet *Latia* (see Sect. 3.4), and copepods [10, 11]. These behaviors will make predators lose track of the prey's path, allowing it to escape safely. However, this strategy is sometimes indistinguishable from that of creating a smokescreen of luminous material in marine environments.

Aposematism is a signal displayed to potential predators to warn of toxicity and/or noxiousness. For example, firefly larvae and adults are both noxious and toxic for various predators, including insects, centipedes, spiders, fish, amphibians, reptiles, and mammals [12–16], thus a function of firefly luminescence has been considered to be an aposematic display [17–19]. All known larvae of lampyrid species are luminous, but the adults of some species are nonluminous [20]. Therefore, it seems that aposematism was a contributing factor in the evolution of bioluminescence in the Lampyridae, and subsequently became a part of the courtship behavior in adults [18, 21].

All known phengodid beetles are also luminous in at least the larval stages and as adult females [8]. Grimaldi and Engel [22] suggested that their bioluminescence functions as an aposematic signal in most species, but as a courtship signal in only some species. A brownish substance discharged from *Phrixothrix* larva is inflammatory to human skin upon contact [23], and *Rhagophthalmus* secretes a caustic odor by stimulation [7, 24]. Toxicity and distastefulness have not been reported in click beetles, including luminous species. Therefore, luminescence in these species is not likely to be aposematic in function.

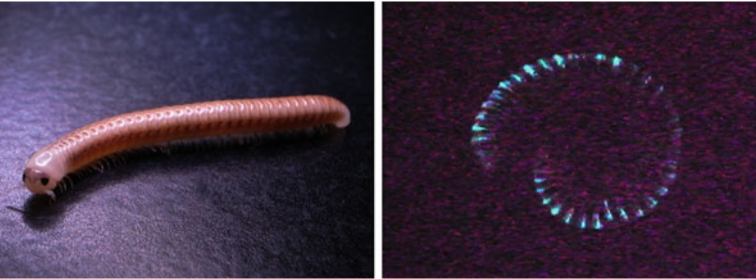

Fig. 5 Luminous millipede *Paraspirobolus lucifugus* collected in Okinawa, Japan. *Blue-green* luminescence was elicited by stimulating with chloroform vapor

The function of the luminescence in millipedes and centipedes has been interpreted as aposematism [25, 26]. The luminous millipedes *Motyxia* spp. gradually intensify the glow of their entire bodies and discharge noxious cyanide-containing secretions when stimulated [26, 27], and a species of *Salpidobolus* similarly sprays caustic substances [28]. When the author stimulated the luminous millipede *Paraspirobolus lucifugus* with forceps, the millipede emitted luminescence from gaps between the body segments along with an unpleasant smell (Fig. 5). Luminous secretions of the centipede *Otostigmus aculeatus* induced erythema and blisters on human skin [29].

Aposematic coloration, as in the cases of poisonous and colorful butterflies of the genus *Heliconius*, usually allows the evolution of palatable mimics (Batesian mimicry) and/or unpalatable mimics (Müllerian mimicry) [30]. It has been known that some cantharid and lycid beetles as well as other insects (at least 20 species belonging to 11 families and four orders of insects) share color patterns with Papua New Guinea lampyrids, *Pteroptyx effulgens* [31]. The mimicry involved in these cases has been interpreted as a combination between Mertensian and Müllerian mimicry; fireflies may usually be distasteful, but not lethal, whereas numerous species especially those belonging to the family Lycidae are considerably more toxic [18, 32]. However, none of firefly-mimics have yet evolved luminescence properties, and there is the possibility that fireflies mimicked some lycids [31] (VB Meyer-Rochow, personal communication).

Brightly colored mushrooms are sometimes poisonous: an aposematic display [33]. Similarly, the persistent glow of bioluminescent mushrooms may be an aposematic signal of unpalatability toward nocturnal fungivores. However, this hypothesis has not been proven [34]. The Japanese luminous mushroom *Omphalotus japonicus* is toxic to humans, but the author (YO) has observed staphylinid beetles frequently consuming this mushroom. On the Japanese island of Hachijo, the luminous mushroom *Mycena chlorophos* is an economically important tourist attraction, but the damage to fruiting bodies by land snails and ants is a problem (Fig. 6).

Although aposematic luminescence occurs in many terrestrial species, this strategy seems to be rare in the ocean. One probable example is the luminous

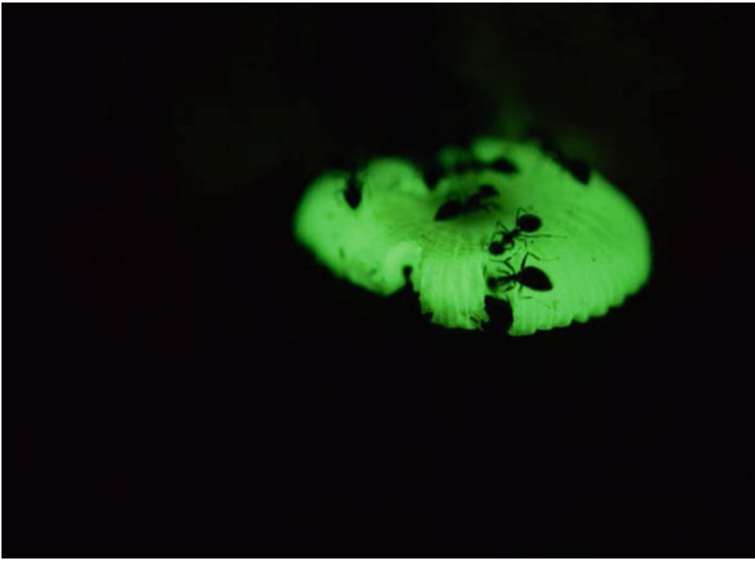

Fig. 6 Luminous fungus *Mycena chlorophos* fruiting body damaged by ants at nighttime in situ on Hachijo Island, Japan. Photo courtesy of Masashi Naito

brittle star *Ophiopsila* (Ophiuroidea, Echinodermata) [35]. Found in shallow reefs, the brittle star *O. riisei* is unpalatable for some potential predacious crabs, and when disturbed by predators it flashes rapidly from its arm-tips. In repeated laboratory trials, the crabs showed increases in avoidance behavior in response to brittle star luminescence [36]. Wilson and Hastings mentioned in their book [37] that ostracods are distasteful, thus the luminescence might also function as an aposematic display. On the other hand, one study showed that ostracods comprised 3–6 % of the gut contents of gobiid fish collected at Odawa Bay of Japan [38], which suggests that ostracods are palatable for some predators. Haddock et al. [2] hypothesized that luminescence in cnidarians also plays a role as an aposematic display, but it has not yet been demonstrated.

In our observations, the Japanese fireworm *Odontosyllis* released luminous mucus when attacked by gobiid fish in the aquarium. Lastly, some specimens were observed breaking off brightly luminescent posterior body segments. A similar behavior was observed during rough handling (Fig. 7). We also observed that some gobiid fishes regurgitated the fireworms, whereupon they began to luminesce brightly. These observations suggest that *Odontosyllis* employs several of these luminescent self-defense tactics: startling, decoy, and aposematism.

Fig. 7 Luminous fireworm *Odontosyllis* sp. after autotomy by stimulation. Detached tail tip glows continuously. Specimens were collected in Toyama Bay, Japan. Photo courtesy of Masashi Naito

2.2 Light Organ Lure

Deep-sea anglerfish, such as *Himantolophus,* are known to attract prey with a light organ created by cultivating symbiotic luminous bacteria in the tips of their escas, that is, the lure-like structure created from the modified first spine of the dorsal fin. However, anglerfish have not been observed in situ luring prey using luminescence. Using bioluminescence to attract prey has also been suggested for other marine animals, such as stomiid dragonfish, *Chiroteuthis* squid, *Stauroteuthis* octopus, and Siphonophores [2].

There are some hypotheses as to why prey are attracted to luminescence in the ocean. One plausible explanation is that luminescent particles in the sea visually indicate food. For example, deep-sea marine snow contains organic detritus and fecal pellets that host glowing bacteria. Small zooplanktons and fry detect the luminescence and consume the luminous material [39], and consequently, zooplankton are attracted to the luminescence. Zarubin et al. [39] suggests that the luminescence of nonsymbiotic bacteria enables them to find a host and to propagate in the nutrient-rich intestines of zooplankton and fish. Previous studies corroborate the high level of nonsymbiotic luminous bacteria in fecal pellets [40, 41].

The caves and riverbanks of Australia and New Zealand are home to larvae of fungus gnats, *Arachnocampa* spp., that attract small phototactic insects with luminescent posterior light organs into nonluminescent snares of vertical threads (termed “fishing-lines”) coated with mucous [42–44] (Fig. 8). A similar behavior

Fig. 8 Luminescence of the carnivorous fungus gnat *Arachnocampa luminosa* on a riverbank near Auckland, New Zealand. Photo courtesy of So Yamashita

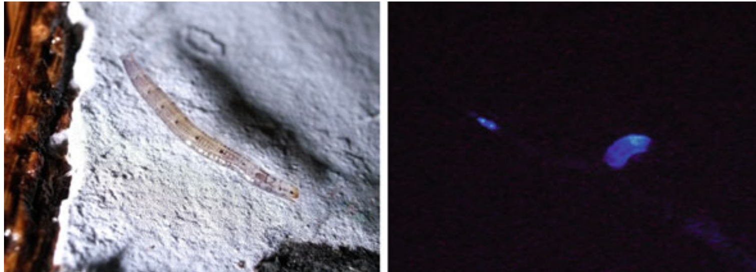

Fig. 9 Luminescence of spore-feeding fungus gnat *Keroplatus nipponicus* on Hachijo Island

was also reported from the North American fungus gnat *Orfelia fultoni* [45, 46]. Interestingly the larvae of fungus gnats of the genus *Keroplatus* (Fig. 9), are also luminescent, despite that these species are likely to be spore-feeders [47]. The luminescence function of *Keroplatus* is unclear [9].

Most fireflies do not feed in adult stages. By contrast, it is very unique and exceptional that female adults of the North American fireflies *Photuris* and *Bicellonycha* mimic flash patterns of other fireflies, for example, *Photinus* and *Pyractomena* females, thereby attracting the males of the latter as prey [48]. *Photuris* cannot produce toxins by themselves, so they obtain the toxins, rather than nutrition, from prey fireflies and use them for aposematic display [49–51]. Larvae of the luminous click beetle *Pyrearinus termitilluminans* reside in termite mounds in Central America, and emit light from their prothorax to attract alate ants and termites as prey [52].

One of the roles of luminescence in mushrooms may be the attraction of spore-dispersing organisms. This hypothesis has been widely accepted for many years, but has never clearly been demonstrated [34].

Luminescence intended for prey sometimes attracts predators instead. The cave harvestmen *Megalopsalis* and *Hendea* possess prominent eyes to detect the luminescence of larval *Arachnocampa luminosa,* which are their major prey [53]. It is hypothesized that some large-sized marine predators such as sperm whales and leatherback turtles may use bioluminescent cues in their search for their food ([2], and references therein).

2.3 Intraspecific Communication

On land, it is well known that some adult fireflies use luminescence signals for sexual communication between males and females [51, 54, 55]. Larviform females of *R. ohbai* display their dorsal photophore toward males with continuous luminescence (Fig. 3), suggesting the function of luminescence is sexual communication [55]. The luminescence of the females of other phengodid beetles, *Dioptoma* and *Diplocladon*, also functions to attract their respective males [54, 56]. The function of luminescence in luminous click beetles has also been suggested as a means of sexual communication between males and females [4, 57]. Indeed, the male adults are sometimes attracted to artificial lights, including cigarettes [58]. The adults of the New Zealand fungus gnat *Arachnocampa luminosa* may use luminescence for sexual communication [42, 59, 60]; this has been challenged by Broadley [61], who provides evidence in support of pheromonal attraction.

In the ocean, bioluminescence in some ostracods, including the genus *Vargula* and the polychaete genus *Odontosyllis* has been suspected to possess a role in sexual communication based on observations of their behavior in the wild [4, 10, 38]. Luminescence of certain shallow water fish, such as leiognathid ponyfish and the flashlight fish *Photoblepharon palpebratus*, may function as a means of intraspecific communication (for mating, dominance display, and/or schooling) [62–64]. Based on some sexual dimorphism in photophores (light organs) it has been suggested that various midwater organisms use luminescence for sexual communication [65, 66].

2.4 Camouflage

Many organisms use bioluminescence to camouflage themselves in the open from potential predators in a strategy called "counter-illumination." This strategy is most prevalent in the marine midwater, a depth down to which dim blue light still penetrates.

In the open ocean it is rare to find objects to hide in or behind, so mesopelagic animals become silhouetted against the seawater-penetrating sun and moonlight to observers below. This silhouette is less advantageous for large animals, such as

Fig. 10 Midwater hatchetfish *Sternoptyx pseudobscura*, with ventral photophores for counter-illumination and large eyes for finding the silhouettes of prey by looking up. Photo courtesy of Laboratory of Marine Biology, Kochi University

turtles, whales, and sharks. On the other hand, smaller animals are at risk of attack for many predators look up to find prey (Fig. 10).

Dahlgren [67] first suggested that ventral light organs in luminous squid are used to negate their silhouette by shining at ambient brightness levels. This counter-illumination hypothesis has also been used to explain luminescence in various luminous fish and shrimp; some of their downward-pointing light organs were shown to dynamically match background light intensity in laboratory studies [68, 69].

Counter-illumination is common in marine mesopelagic dwellers [70] but not supported in terrestrial and freshwater species. The myriad shelters and hiding spots on land as well as in the shallow or cloudy waters of rivers, ponds, and lakes may in the course of evolution have led to alternatives to bioluminescence as a survival strategy.

2.5 Searchlight

Species in three genera of deep-sea dragonfish, *Malacosteus, Aristostomias*, and *Pachystomias,* emit red light from a pair of photophores under the eyes. This unusual red, postorbital luminescence is considered to be akin to "night-vision" illumination. Because most deep-sea organisms are adapted to blue-light sensitivity for dim, blue downwelling light and bioluminescence detection [71], they are essentially blind to the red light projected by dragon fish. Indeed the vision of *Malacosteus niger* is red light sensitive, so it can seek and find unsuspecting prey [72, 73]. The anomalopid flashlight fish possess a pair of large suborbital light organs under their eyes, for which Morin et al. [62] have suggested a function to find and/or attract prey and Meyer-Rochow et al. [74] have reported an increased light emission activity in *Anomalops katoptron* during feeding. However, it is difficult to distinguish whether the photophores located near the eyes and the mouth function biologically as decoys or searchlights.

2.6 Functionless Bioluminescence

Visible light from some organisms may not have any significance in itself. Bioluminescence is considered to be an energetically low-cost mechanism [75, 76], and thus can potentially evolve as a metabolic byproduct unless the light emission is disadvantageous. For example, it has been proposed that fungal luminescence is a byproduct of some unknown metabolic process [34, 77]. Luminescence of organisms emitting very weak light, such as the Japanese fungus gnat *Keroplatus nipponicus*, or glowing underground, such as the annelid *Microscolex phosphoreus*, may also be functionless.

3 Terrestrial Taxa

Because of its accessibility to observers, bioluminescence in terrestrial habitats has received a great deal more attention ecologically and taxonomically than that of aquatic habitats. Terrestrial environments are varied, and in the following we discuss various luminous organisms' life histories and physiologies related to their habitats: land, air, underground, and freshwater.

3.1 On Land

Terrestrial luminous organisms comprise arthropods and fungi. One exception is the world's only known luminous terrestrial mollusk *Quantula striata* (syn. *Dyakia striata*) of Southeast Asia [78]. These land snails, especially the young snails, produce intracellular light on or near the mouthparts, but do not respond to disturbance. The biological and ecological significance of the luminescence in these land snails is uncertain, but it has been proposed to facilitate aggregation behavior [79]. In the Plantae, no luminous species have been discovered. *Schistostega pennata* (syn. *Gymnostomum pennatum*), generally called "luminous moss," is not autoluminescent: it appears to glow emerald green or gold on cave floors, but the glow is actually a directional reflection of dim light by a particular cell structure of the protonema [80]. No luminous species are known from tetrapods (amphibians, reptiles, birds, and mammals), instead they can use many other remote sensory and communication systems; sound, odor, vibration, color vision, UV, thermal radiation, ultrasonic, and even electricity.

Among the arthropods, the Myriapoda and Hexapoda contain luminous species, but no luminous species are found in another large arthropod subgroup that includes spiders and scorpions, the Chelicerata. Given that the larvae of the fungus gnat, *Arachnocampa*, lure positively phototactic prey into their curtains of sticky

vertical threads using luminescence, it seems strange that no luminous spider has been reported to date.

In the subphylum Myriapoda, some millipedes (Diplopoda) and centipedes (Chilopoda) emit light, with 24 known species altogether [25]. In the subphylum Hexapoda, luminous species are only found in elateroid beetles (Coleoptera), fungus gnats (Diptera), and springtails (Collembola) [9, 81].

Coleoptera are the largest order in the class Insecta, containing about 350,000 species in over 160 families worldwide [22]. Luminescent species of beetles are found in three elateroid families: Lampyridae, Phengodidae, and Elateridae [81, 82]. All species of the Lampyridae and Phengodidae (roughly 2,000 and 200 species worldwide, respectively) are luminous, and of the approximately 10,000 elaterid species worldwide, approximately 200 are luminous [83].

The Diptera (true flies) are the fourth largest order in the Insecta, containing 150 families and 150,000 species [84]. Despite their huge diversity, only a small number of luminous species ($\sim$15 species) are known from a single family, the Keroplatidae [9]. The bioluminescence mechanism of *Arachnocampa* fungus gnats is considered to be different from that of *Orfelia* [85], but the details are still unknown.

In Collembola, luminescent species have been recognized in the families Neanuridae and Onychiuridae [4, 56], but the observation records are limited. The author (YO) observed the luminescence of *Lobella* sp. (Neanuridae), in which the specimens emitted a continuous weak light from the body after stimulation with forceps. The function of luminescence in *Lobella* sp. is uncertain but it seems too faint to deter predators or to use for intraspecific communication [9].

Recently luminescence was claimed to occur in some South American cockroaches (Blattodea) [86, 87], but the veracity of these reports is highly dubious [88].

It is interesting that Lepidoptera, the second largest order in Insecta [22] apparently possess no luminous species. Most adult moths are nocturnal and some are poisonous; thus bioluminescence would seem a suitable tool for communication by moths. However, lepidopteran insects do not seem to have evolved any bioluminescence and instead widely use pheromones for their nocturnal communications. It seems worth noting that some nonluminous fireflies also use pheromones for intraspecific communication, but in contrast to the moths, the beetles are diurnal. In Hemiptera, the fifth largest insect order [22], the elongated head of the lantern fly *Fulgora laternaria* in South America had long been believed to be luminescent, but this has now become very doubtful [89].

Luminous species of the three elateroid beetle families use the same luciferin molecule in their luminescence mechanism, (4*S*)-4,5-dihydro-2-(6-hydroxy-2-benzothiazolyl)-4-thiazolecarboxylic acid, and a homologous luciferase sharing over 48 % amino acid identity [90]. Phylogenetic relationships among these families have been studied in the context of the evolution of bioluminescence [18, 91], but are still not fully resolved. Recent molecular analysis based on the mitochondrial genome revealed that Lampyridae and Phengodidae are sister groups in the cantharoid beetles [92], suggesting a single origin of their luminescence. Although Elateridae are also closely related to the Lampyridae–Phengodidae clade [92],

phylogenetic analyses of Elateridae showed that luminous click beetles are deeply nested within the nonluminous species, thus bioluminescence may have arisen independently in Elateridae [93, 94]. These results indicate that bioluminescence in beetles may have evolved once or twice [81].

Bioluminescence mechanisms of the land snail *Quantula*, millipedes, centipedes, fungus gnats, and springtails are still unknown in detail ([9], and references therein), but probably are independent from each other. Therefore, this author (YO) suspects that bioluminescence has evolved at least seven times among terrestrial organisms, including the case of luminous fungi described hereafter.

Luminous fungi are found only in the phylum Basidiomycota (Division Eumycota, sensu Parker [95]), that is, the taxon of fungi that produces large, apparent fruiting bodies referred to as “mushrooms” (approximately 32,000 species [96]). About 70 species of luminescent fungi have been recognized worldwide, and they belong to three different subclades: *Omphalotus*, *Armillaria*, and the mycenoid fungi [97]. Although all fungal luminescence is intracellular, some species glow only in the mycelia (e.g., *Armillaria* species), whereas others luminesce in both mycelia and fruiting bodies (e.g., *O. japonicus* and *M. chlorophos*). Even though the bioluminescence mechanism of the luminous fungi is not fully understood [98], the reaction mechanism is known to be identical in all species [99]. The function of bioluminescence in fungi has long been questioned, and remains unconfirmed experimentally.

Terrestrial luminescent bacteria of the genus *Photorhabdus* adhere to the intestine of a *Heterorhabditis* nematode worm: the nematode burrows into its insect host and regurgitates *Photorhabdus* into the hemocoel; the bacteria grow in the insect body and finally kill the insect. The Nematode feeds exhaustively on the bacteria or products of bacterial degradation of the hemolymph then leaves the cadaver in search of a new host insect. The luminescence of *Photorhabdus* is observed only when it grows in the insect cadaver. The ecological functions of the cadaver luminescence has been uncertain, but one suggestion was that it may represent an aposematic warning to nocturnal scavenging animals or act as a lure to attract further insect prey [100].

3.2 In the Air

Some lampyrid beetles are well known for luminescing during flight, and are rightly commonly called fireflies. However, not all luminous beetles are capable of flight. Some phengodid (e.g., *Rhagophthalmus*) and lampyrid beetles (e.g., *Lampyris*) have luminous but flightless wingless females (Fig. 3) and are commonly called glow-worms, whereas the males are capable of flight (winged) but are nonluminous or only very dimly luminescent [20, 55]. Male and female adults of some lampyrid genera are able to fly but are nonluminous, such as *Lucidota*, *Pyropyga*, and *Pristolycus* [101]. The manipulation of luminescence in some winged fireflies, such as in *Luciola* and *Photinus*, is very sophisticated: they use

species-specific flash signals to recognize conspecific males and females [51, 82, 101]. All luminous click beetles are likely capable of flight as adults, and it has been suggested that these males and females use the light for sexual communication while flying [57, 101].

3.3 Underground: Why in Soil?

In the Oligochaeta (earthworms and potworms), luminous species have been reported from 16 genera worldwide [9, 102]. Why do these underground dwellers produce light? The answer remains unclear, but it is considered to play a defensive role against predators [102]. Luminous earthworms discharge luminescent mucus from the mouth, the anus, and/or the body wall upon mechanical stimulation [102]. These earthworms are potentially palatable: *M. phosphoreus* was consumed by various potential predators, such as an earwig, a spider, a mole cricket [103], a topminnow, a newt, and a lizard (Oba, unpublished). The New Zealand giant luminous earthworm *Octochaetus multiporus* was eaten by a kiwi bird without ill effect (VB Meyer-Rochow cited in [102], and personal communication); *M. phosphoreus* captured by a centipede (Lithobiidae gen. sp.) was observed at nighttime in nature (Fig. 11; H Yoshida, personal communication).

These observations indicate that the light of luminous earthworms may be to startle, but not to aposematically warn predators in the darkness underground. Sivinski and Forrest [103] fed *M. phosphoreus* to mole crickets, an underground-dweller that feeds on earthworms. A mole cricket immediately captured the worm, but dropped the worm from its grasp and drew away when the worm discharged luminous mucus. Finally, this mole cricket attacked the worm again and consumed it, but this observation suggests that the luminescence of earthworms may provide the prey with some opportunity to escape from the predator.

Pupae of some lampyrid species, such as *Luciola*, produce underground mud cocoons and emit a dim green light from their entire body. When disturbed, they also emit an intense yellow light from their ventral photophore (Fig. 12). The biochemical mechanism of the dim glow in lampyrid pupae is understood [104], but the biological advantage of the dual colored luminescence remains unknown.

3.4 Fresh Water: Why so Rare?

Compared with the rich diversity of luminous organisms in the sea, luminous freshwater dwellers are quite rare. Fish and crustaceans make up a large percentage of the list of luminous organisms [1, 105], but there are apparently no luminescent freshwater fish and crustacean species.

The limpet *Latia neritoides* (Latiidae, Mollusk) found in streams of New Zealand's North Island is the world's only luminous freshwater gastropod species.

Fig. 11 Luminous earthworm *Microscolex phosphoreus*, attacked by the lithobiid centipede at night. Photo courtesy of Hiroshi Yoshida

Fig. 12 Luminescence of the Japanese firefly *Luciola lateralis* male pupa. The light organ on the abdominal segments VI and VII emits *yellow light* upon stimulation. The rest of the body continuously emits *green light*. Two independent luciferases are involved in this phenomenon

This small basommatophoran snail clings to the surface of submerged stones (Fig. 13) and discharges green-yellow, brightly luminescent mucus when disturbed. Meyer-Rochow and Moore [106] reported that the luminescent mucus was released into the stream when the limpet was attacked by predators, such as fish,

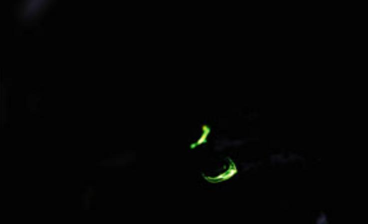

Fig. 13 Luminous freshwater snail *Latia neritoides* discharging luminescent mucus by stimulation at the river near Auckland, New Zealand (same as the place for Fig. 8). Photo courtesy of So Yamashita

crayfish, or dragonfly larvae, and suggested "the predators may chase after luminescent droplets washed away from *Latia* instead of attacking *Latia* itself".

Most of the lampyrid species are terrestrial for all phases of their lifecycle, but only a few *Luciola* species (~10 species) are known to be aquatic during the larval stage [107]. They adapt well to the aquatic environment where they locate and overpower freshwater snails in order to feed on them. Most, but not all, of the aquatic species' larvae respire in water using tracheal gills [16, 107]. These aquatic larvae project white glands from abdominal segments that emit a fetid smell, suggesting that the luminescence of some aquatic larvae is also an aposematic display to predators, such as freshwater goby and dragonfly larvae [14].

4 Marine Taxa

Although there are various terrestrial luminous organisms, they nevertheless belong to only a few taxonomic groups. This contrasts with marine taxa, in which luminous species are distributed across most of the large taxonomic groups, except for diatoms, marine macro-algae, and sponges (see [2]). In total, about 80 % of the genera containing luminous species are marine dwellers [1, 105].

4.1 Intertidal Zone

The intertidal area between the sea and land, called the littoral zone, is regularly exposed to variable conditions: rain causes low salinity, and sunlight causes heat, desiccation, strong UV/visible light, and high salinity. Only a few shellfish and an earthworm are reported as luminous in this environmentally challenging zone.

Some tiny luminous gastropod snails, *Angiola* and *Hinea* (Planaxidae), can be found under rocks in tide pools at low tide and on stony beaches [108–111]. The luminous bivalve *Pholas dactylus* inhabits the shallow sublittoral zone of soft-rock coasts, typically gneiss [4]. It is of interest that the luminous bivalves and

Fig. 14 Luminous marine snail *Angiola zonata* at stony beach of Hachijo Island, Japan. These specimens were found aggregated after upturning the pictured stone

gastropod snails are found only in the harsh intertidal zone, given that there are many thousands of related species that inhabit almost every depth of the ocean. One possible exception is the eulimid gastropod snails *Melanella luminosa,* which was found as a parasite on the scarlet sea cucumber at a depth of 10–238 m; Marshall [112] has described its yellow glow.

In the snail *Angiola zonata* (syn. *Angiola longispira*) (Fig. 14), a patch on the mantle (or hypobranchial gland, [109]) emits a weak blue-green light when the animal is stimulated mechanically or exposed to high salinity ([108], S Yamashita, personal communication). Houbrick [109] reported that all species in *Angiola* appear to be nocturnal and suggested that the light in "*Angiola* species may have a startling effect on predators". Deheyn and Wilson [111] suggested that an aposematic function "is less probable considering the gregarious and cryptic behaviour" of planaxid snails. The author (YO) observed in aquaria that fry of the intertidal teleost *Kuhlia mugil* consumed the soft bodies of *A. zonata* with no apparent ill effect, suggesting the snail's luminescence is not for aposematic display. In *Pholas*, luminescent mucus is discharged from the siphon when disturbed, and as Harvey suggested, "the best guess is that the light masses scare away predacious animals" [4], but the actual function remains uncertain [37].

The luminous earthworm *Pontodrilus litoralis* (syn. *P. matsushimensis*, *P. bermudensis*) inhabits the littoral zone of sandy beaches. The function of bioluminescence in *P. litoralis* remains unclear, likewise in other luminous earthworms. In Japan, this species is sometimes used as bait for fishing [113]. Furthermore, a crab and goby immediately fed on living *P. litoralis* when introduced into aquaria, and no apparent ill effects in the predators were observed after consuming the luminescent earthworm, suggesting it is neither distasteful nor toxic for potential predators. The author (YO) has observed carnivorous insects such as earwigs (Dermaptera) and staphylinid beetles (Staphylinidae) occurring sympatrically with *P. litoralis*. These observations suggest that the function of *P. litoralis* luminescence might be to startle, but not as an aposematic display. On the other hand, as luminous fluid is discharged only when *P. litoralis* specimens are severely stimulated, such as being cut, squeezed, and/or beaten [9, 114], the startling hypothesis will need to be verified.

4.2 Coastal Water

In the neritic zone, the number of luminescent species is relatively low, a reported 1–2 % of all coastal species [115]. However, each luminous species often occurs in high density in its environment. This situation is in contrast to those in oceanic waters where species diversity is very high, but population density of individual species is usually low (see Sect. 4.3). Luminous species usually make up only a small proportion of a phylum's total number of species, but there are two exceptions: the Cnidaria (jellyfish) and the Ctenophora (comb jelly). A large proportion of the former phylum and nearly all of the latter are luminous [4], and these two groups are common members of the coastal planktonic dwellers. As for the biological function of luminescence in coastal dwellers, Morin [115] suggested that these species primarily use fast flashes of luminescence to deter prey or continuously glowing decoys to distract predators, giving the prey an opportunity to escape.

Dinoflagellates (Division Chromophycota, sensu Parker [95]) are important primary producers in coastal waters. Some of the free-living species are luminous, and most of these are autotrophic and therefore live in shallow waters where they can photosynthesize. The luminous dinoflagellate *Noctiluca scintillans* is an obligate heterotroph, but it also lives in shallow water to feed on autotrophic diatoms, other dinoflagellates, and even small zooplankton [37]. For the enzymatic bioluminescence reaction, luminous dinoflagellates use open-chain tetrapyrrole luciferin [116], which is structurally related to chlorophyll. Interestingly, luminous krill (Euphausiidae) also use the tetrapyrrole luciferin, which is structurally very similar to the luciferin of dinoflagellates [117, 118]. Accordingly, it is expected that krill consume luminous dinoflagellates and chemically modify the dinoflagellate luciferin as a precursor to their own luciferin [119]. To date, other luminous taxa that use dinoflagellate luciferin or its derivatives as a luminous substrate have not been reported.

4.2.1 Cypridinid Luciferin

In ostracods, luminous species in the genera *Vargula* and *Cypridina* are coastal, whereas species in the genus *Conchoecia* are midwater dwellers [120]. The two former genera use cypridinid luciferin (also called Cypridina luciferin) as a bioluminescent substrate [98, 121], whereas *Conchoecia* use coelenterazine [98, 122]. These findings are consistent with the fact that no midwater fish are known that use cypridinid luciferin as a bioluminescent substrate (see below).

Coastal fish with nonbacterial luminescence rely on cypridinid luciferin in bioluminescence reactions [123, 124]. These types of luminous fish are found in three distantly related families of the orders Perciformes and Batrachoidiformes [123–128]: *Parapriacanthus ransonneti* (syn. *P. beryciformes* [129]) and *Parapriacanthus dispar* (Pempheridae); *Pempheris ypsilychnus* (Pempheridae [130]);

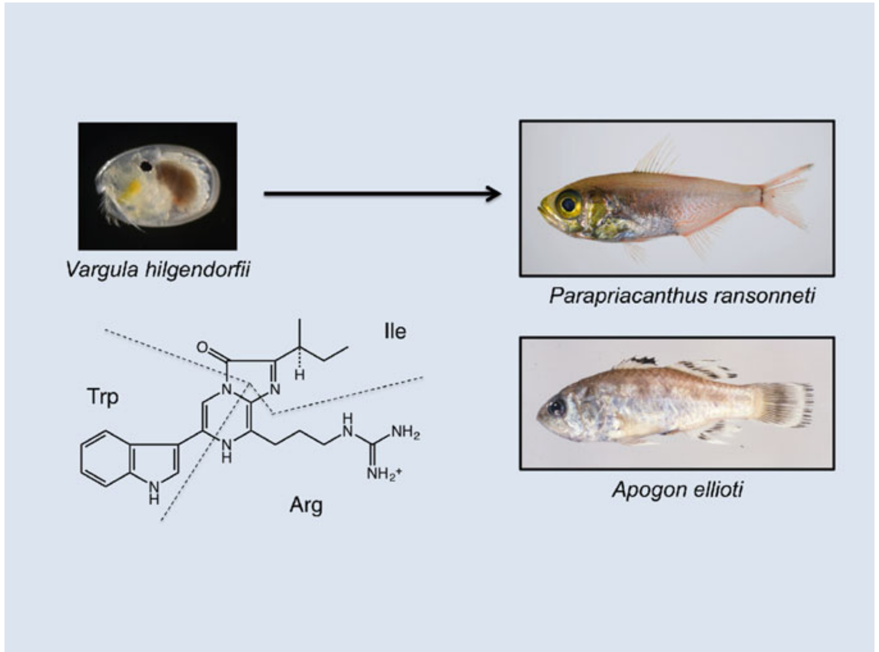

Fig. 15 In the marine coastal zone, cypridinid luciferin is biosynthesized by ostracods. It is considered that some coastal fish consume the ostracods and obtain the cypridinid luciferin for their own bioluminescence reactions. Photos courtesy of Ken-ichi Onodera (*Vargula*), and the Laboratory of Marine Biology of Kochi University (*Parapriacanthus* and *Apogon*)

Apogon ellioti (syn. *Jaydia ellioti*) and *Apogon poecilopterus* (syn. *Jaydia poecilopterus*) (Apogonidae); midshipman fish *Porichthys* spp. (*P. notatus*, *myriaster*, and *porosissimus;* Batrachoididae); and presumably some *Archamia* and *Rhabdamia* species (Apogonidae).

It has been suggested that these coastal fish obtain cypridinid luciferin by consuming *Cypridina* and *Vargula* ostracods [37]. In fact, ostracod specimens have been discovered from the stomach of *P. ransonneti* ([131], Oba and Hiruta, unpublished), and a nonluminous form of *P. notatus* recovered its luminescent ability following injection of cypridinid luciferin [132]. Recently, the author (YO) and colleagues demonstrated that cypridinid luciferin is biosynthesized from three amino acids (Ile, Arg, Trp) in *Vargula hilgendorfii* (Fig. 4) and *Cypridina noctiluca* [133–136]. These findings indicate that cypridinid luciferin produced by coastal ostracods partly contributes to the species' richness of luminous fish in the coastal environment (Fig. 15). Indeed, cypridinid luciferin chemiluminesces strongly in certain solvents, such as diglyme, dimethyl sulfoxide, and detergent-water solution, without an enzymatic catalyst [137]. This high reactivity suggests that cypridinid luciferin is potentially a good bioluminescent substrate by nature. Therefore, it may not be difficult to evolve luciferase from an extant protein, if organisms are able to obtain cypridinid luciferin by diet.

4.3 Midwater: Why so Species-Rich?

Previous research suggests that nearly all of the luminescent species known to occur in the midwater of oceans. Beebe [138] estimated that based on a study in Bermuda that luminous mesopelagic fish collected below a depth of 600 m represent 81 % of the families, 66 % of the species, and 97 % of all the individuals. Calanoid copepods, krill, and *Cyclothone* fish represent a large part of total macro- and microzooplanktonic marine biomass, and these taxa contain particularly many luminous species [4].

Haddock et al. [2] listed hypotheses of why most luminescent taxa are ocean dwellers: they suggested that the sea is favorable for the evolution of bioluminescence because it is (1) a stable environment, (2) optically clear relative to other aqueous habitats, (3) continuously dark or very dim, and (4) highly diversified taxonomically with complex interspecific relationships. We suggest that there are additional factors that favor the evolution or acquisition of bioluminescent capabilities in marine organisms: namely "symbiotic luminescence" and "dietary supply of coelenterazine".

4.3.1 Symbiotic Luminescence

Luminous bacteria are common in seawater, especially in temperate and coastal oceans, but they are rarely found in freshwater or on land [139]. Accordingly, marine animals constantly contact, ingest unintentionally, or feed on luminous bacteria. Therefore, it is reasonable to assume that symbiotic luminescence could have evolved independently several times in various fish and squid.

There are at least seven orders of fish and two orders of squid (orders Sepiolida and Teuthida) that employ luminous bacteria in both coastal shallow water and midwater [139–141] (Fig. 16).

Species of luminous bacteria found in symbiotic hosts living in shallow water are different from those in midwater. Shallow-water fish, such as leiognathid ponyfish and pinecone fish, and bobtail squid *Euprymna* and *Sepiola* use *Photobacterium leiognathi*, *P. mandapamensis, Aliivibrio fischeri*, *A.* "*thorii*," or *A. wodanis* (formerly *Vibrio fischeri* and *V. logei* [139, 142]). In contrast, midwater species, such as chlorophthalmid and macrourid fish, predominantly use *Photobacterium kishitanii* (formerly included in nonsymbiotic *P. phosphoreum*) [139–141]. The symbiotic bacteria obtain essential nutrients from their hosts and begin to luminesce less and less, or even die, when the host is starved [143].

Considering the advantages of bioluminescence for marine life forms, it is surprising that only fish and squid evolved mutual symbioses with luminescent bacteria. It has been suggested that the bioluminescence of one species of the pelagic colonial tunicate *Pyrosoma* is due to bacterial symbiosis, but details are not available [98].

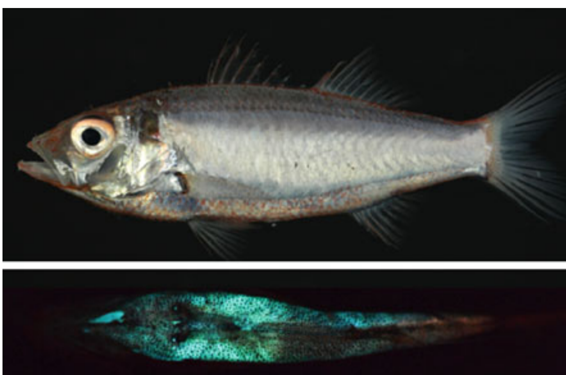

Fig. 16 Luminous coastal fish *Acropoma japonicum* (Acropomatidae) harboring the luminous bacteria *Photobacterium leiognathi* and *P. mandapamensis*, and its ventral bioluminescence. Photo courtesy of Laboratory of Marine Biology of Kochi University

4.3.2 Coelenterazine

Coelenterazine was originally named for the bioluminescent substrate found in coelenterates (Cnidaria and Ctenophore), but the term is still used despite the wide variety of marine taxa that use the same molecule for their bioluminescence reactions [98]. Organisms that use coelenterazine or its derivatives for their bioluminescence are scattered at least in five phyla: the Cnidaria, Ctenophora, Mollusca (e.g., the squid *W. scintillans* and *Symplectoteuthis luminosa*), Arthropoda (e.g., ostracod *Conchoecia*, copepods, and the decapod shrimps), Chordata (e.g., myctophid lanternfish [98]), and putatively in the Echinodermata (the benthic brittle star *Amphiura filiformis* [98]), Chaetognatha (the bathypelagic arrowworm *Caecosagitta macrocephala* [144]), and the Radiolaria (solitary spumellarian *Thalassicolla* sp. [145]; Phylum Sarcomastigophora, sensu Parker [95]). In midwater, fish of the family Myctophidae are very abundant, and Beebe [138] found in study of Bermuda that approximately one-quarter of the fish species caught by net below 600-m depth were in the Myctophidae. From the view of total specimen quantity, the gonostomatid *Cyclothone* is extremely abundant in midwater [138, 146], and the involvement of coelenterazine in their luminescence has been suggested [147]. On the other hand, there are no freshwater or terrestrial organisms that use coelenterazine or its analogues in bioluminescence. This unusually widespread and abundant recruitment of coelenterazine in marine bioluminescent systems may be a key to understanding the species richness of marine luminescent organisms, and pose questions as to its biosynthetic origin.

Some luminous animals that use coelenterazine in their luminescent systems are not capable of biosynthesizing it. For example, the mysid shrimp *Gnathophausia ingens* and the jellyfish *Aequorea victoria* lost their luminescence after a prolonged period of a coelenterazine-free diet, but recovered luminescence when fed food containing coelenterazine [148, 149]. These findings suggest that some luminous organisms need a supply of coelenterazine from their diet for their luminescence abilities. Thomson et al. [150] concluded that the luminous midwater shrimp *Systellaspis debilis* is capable of coelenterazine biosynthesis on the basis of their

observation that the content of coelenterazine increased during the time in development between newly laid eggs and just before hatching. As Shimomura [98] mentioned, however, their analysis cannot eliminate the possibility that coelenterazine is sequestered as an inactive form in the eggs and converted to active coelenterazine during development; thus coelenterazine may not be biosynthesized de novo in the egg.

Buskey and Stearns [151] showed that the bioluminescence potential of the marine calanoid *Metridia longa* was not reduced even after starvation for 3 weeks. Furthermore, they demonstrated that the bioluminescence potential in *M. longa* fully recovered within 10 h after luminescent substances were exhausted by mechanical stimulation. As luminous copepods use coelenterazine for their luminescence, these findings strongly suggested that *M. longa* can biosynthesize coelenterazine. Recently, we demonstrated for the first time that coelenterazine is biosynthesized in the midwater calanoid *Metridia pacifica* from three amino acids (Tyr, Tyr, Phe) by feeding specimens stable isotope-labeled compounds and analyzing the incorporation of the labeled compounds in coelenterazine by LC-ESI-TOF/MS analysis [152].

Calanoids are one of the most abundant biomass components in the sea and are important prey for marine carnivorous organisms [153], including luminous "coelenterazine-users," such as chaetognaths, myctophid, and gonostomatid fish [154]. Another luminous coelenterazine-user *Thalassicolla* is a unicellular radiolarian, which is known to capture and ingest living copepods [155]. Unknown copepods were found in the stomach of the myctophid lanternfish *Diaphus watasei* (Oba, unpublished). Species of the genus *Metridia* are widely distributed in the oceans and occur in Antarctic waters [156] as well as in the subarctic Pacific Ocean [154], where *Metridia pacifica* is known as a substantial member of the zooplankton biomass. In Toyama Bay in the southern Sea of Japan, *M. pacifica* contributed 37 % (annual mean) to the total copepod biomass [157], and is one of the major prey items for the luminous firefly squid *W. scintillans* (Fig. 2) [158]. Coelenterazine was also present in various nonluminous marine plankton-feeding fish and shrimps but was not detected in benthic scavengers, such as lobsters and crabs [98]. Taken together, it is conceivable that various marine luminous organisms obtain coelenterazine by consuming *M. pacifica* and other copepods, and for this reason, luminous organisms are abundant in the ocean, especially in midwater (Fig. 17).

It has been known that coelenterazine exhibits chemiluminescence in various substances, including egg yolk, BSA, and surfactants [98, 159], indicating that coelenterazine has innate beneficial properties as a bioluminescent substrate. Indeed, coelenterazine-type luciferases and photoproteins have been identified from deep-sea shrimp *Oplophorus*, copepods, the sea pansy *Renilla*, and jellyfish, but do not share significant amino acid sequence homologies [98]. This suggests that luciferases or photoproteins using coelenterazine as a substrate or chromophore have independently evolved from unrelated proteins several times in an example of convergent evolution.

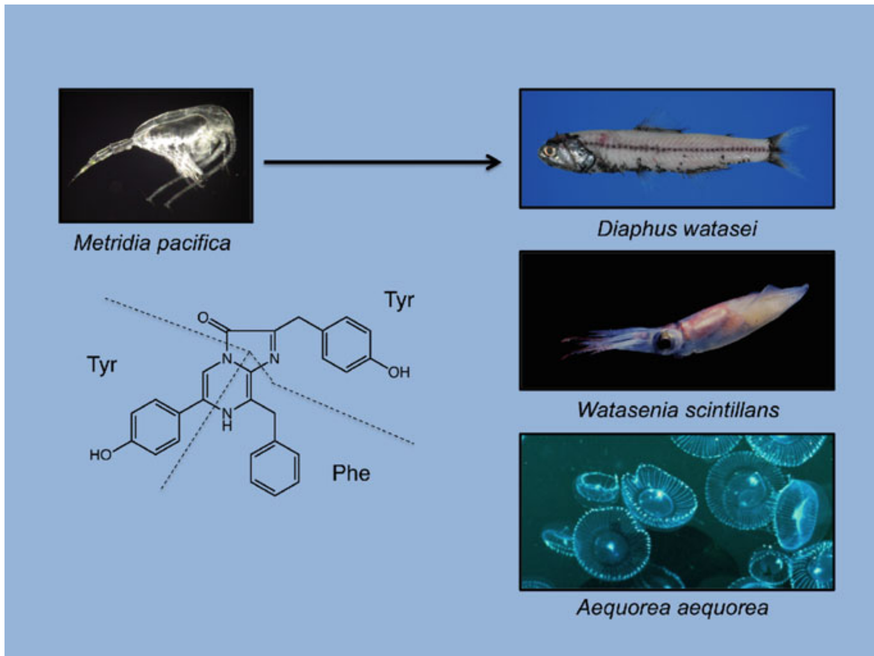

Fig. 17 In the marine midwater, coelenterazine is biosynthesized by copepods. It is considered that various midwater organisms consume the copepods and obtain the coelenterazine for their own bioluminescence reactions. Photos courtesy of Ken-ichi Onodera (*Metridia*), the Laboratory of Marine Biology of Kochi University (*Diaphus*), the Uozu Aquarium (*Watasenia*), and Osamu Shimomura (*Aequorea*)

4.4 Deep-Sea Benthos

Based on a recent study in the Bahamas, the benthic zone of the deep sea (500–1,000 m depth) is not as species-rich in luminous organisms as the midwater zone: less than 20 % of the collected species emitted light [160]. Johnsen et al. [160] explain that visibility in benthic water is obstructed by particulate matter stirred by a bottom current, which is in contrast to the optically clear midwater that facilitates the evolution of bioluminescence [2].

In the deep-sea benthos, luminous species are found mostly in bamboo corals, sea anemones, sea pens, sea cucumbers, and brittle stars [160]. Because it is extremely difficult to observe natural behavior of the organisms, the functions of their luminescence are still unclear. There are many bottom dwellers in crabs, hermit crabs, lobsters, and isopods, but no luminous species have been reported in these groups. An unidentified luminous sea anemone was found on the shell inhabited by a hermit crab [160]. It is known that some sea anemones and hermit crabs engage in a mutualistic symbiosis [161]. If the deep-sea hermit crab positively selects luminous sea anemones as a symbiont, the hermit crab should be

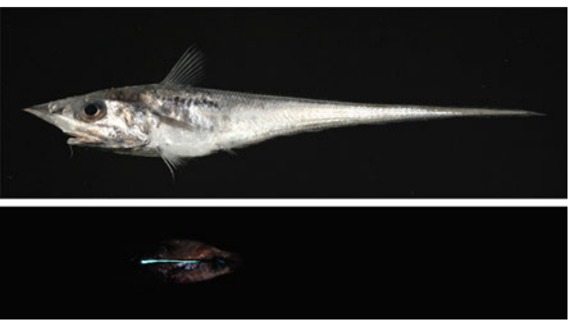

Fig. 18 The deep-sea benthic rattail fish, *Coelorhynchus kamoharai*. The ventral *midline* glows *blue-green* by harboring the luminous bacterium *Photobacterium kishitanii* inside photophores there. Photo courtesy of the Laboratory of Marine Biology of Kochi University

regarded as a luminous organism, as other taxa with luminous symbiotic bacteria are themselves considered luminous organisms.

The rattail fish (Macrouridae) are a large family of teleosts that generally inhabit deep-sea benthic habitats. Most of the species are luminous [162] and have saclike photophores containing luminous bacterial cultures around the anus and along the midline of the abdomen (Fig. 18).

The morphological features of the photophores are an important characteristic for identifying species in the Macrouridae, but whether they use the light of their ventral photophores for intraspecific recognition and communication in the dark is unknown.

As the deep-sea benthos remains a largely unexplored frontier, the story of bioluminescent life there is likely still hidden in the depths.

5 Conclusion

World-leading authorities of bioluminescence, including Newton Harvey (1887–1959), as well as the Japanese pioneers, Sakyo Kanda (1874–1939) and Yata Haneda (1907–1995), focused in their comprehensive works on species lists, habitat, morphology, histology, physiology, biochemistry, biophysics, and evolution of the world's luminous organisms, but scarcely worked on the biological significance of the light [4, 56, 163]. This may be simply due to the difficulty of assigning a definite biological function to the light [164]. However, in some cases the luminescent functions of some organisms are exceptionally well identified. For example, the flash signals of fireflies used for sexual communication [51, 54, 55], the continuous glow of the cave glowworm *Arachnocampa* for prey attraction [165], and the counter-illumination of various midwater dwellers [65] are all well understood. Even in the case of fireflies, however, the functions of synchronous flashing in South Asian species [166, 167], the weak luminescence in diurnal species [55], and the glowing of eggs and pupae [104] are still not resolved. The significance of bioluminescence has sometimes been presumed to be for self-defense, because light emission can be produced by prodding or other forms of

physical stimulation, or the light may be for sexual communication given that morphological variations of photophores between sexes are rather common. It is, however, best to be wary of cursory postulations: for example, stimulation-induced lights may be too weak to startle predators and photophore sexual dimorphism may be involved in behaviors other than mating. Meanwhile, some luminous organisms possess highly sophisticated light organs (equipped with lenses, color filters, reflectors, diffusers), and are neuronally controlled [168], suggesting that the light must have some biological significance, albeit poorly understood. Moreover, some unique hypotheses such as "predatory counter-illumination" for luminous sharks [169], and "luminescent burglar alarms" [115, 164] for luminous dinoflagellates and luminous fungi ([170] and [34], respectively) may illuminate wealthiness of bioluminescence when tested experimentally in future.

6 Perspectives

This chapter has presented an ecological and evolutionary overview of the many divergent bioluminescent taxa found in the world. One important observation has been the great diversity of bioluminescent substrates and enzymes that has arisen during the evolution of the varied taxa. Moreover, the paths of convergent evolution have yielded a wide variety of ecological behaviors that utilize bioluminescence.

Although the luciferin structures of many organisms were resolved between the 1960s and 1980s, many luciferase or photoprotein genes remain unknown, such as those of euphausiid krill *Euphausia pacifica*, the firefly squid *W. scintillans* (Fig. 2), the midwater lanternfish of the family Myctophidae, the shallow water fish *Parapriacanthus* and *Porichthys*, the earthworm *Diplocardia longa*, and the freshwater limpet *L. neritoides*. Several taxa still have eluded identification of both their luciferin and their luciferase, including the millipedes *Paraspirobolus* and *Motyxia*, the polychaete *Odontosyllis*, and the brittle star *Ophiopsila* (summarized in [98]). In the case of luminous fungi, enzymatic involvement in light production is still unresolved [98, 171].

To facilitate future research of bioluminescent organisms, my group is proceeding with a DNA barcoding initiative to create a genetic reference library for bioluminescent organisms, per Hebert et al. [172]. The database will provide a resource for other scientists in the identification of luminous organisms with unresolved bioluminescent systems. Given the many applications of bioluminescent systems using only a few well-characterized protein families, elucidating currently unknown proteins, genes, and substrates will expand the toolkit of molecular science and bioengineering. As there are likewise many species for which the biological and ecological function of bioluminescence is poorly understood or disputed in the literature, this same principle will apply to ecologists who seek to study bioluminescent organisms.

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Biochemistry and Genetics of Bacterial Bioluminescence

Paul Dunlap

Abstract Bacterial light production involves enzymes—luciferase, fatty acid reductase, and flavin reductase—and substrates—reduced flavin mononucleotide and long-chain fatty aldehyde—that are specific to bioluminescence in bacteria. The bacterial genes coding for these enzymes, *luxA* and *luxB* for the subunits of luciferase; *luxC*, *luxD*, and *luxE* for the components of the fatty acid reductase; and *luxG* for flavin reductase, are found as an operon in light-emitting bacteria, with the gene order, *luxCDABEG*. Over 30 species of marine and terrestrial bacteria, which cluster phylogenetically in *Aliivibrio*, *Photobacterium*, and *Vibrio* (*Vibrionaceae*), *Shewanella* (*Shewanellaceae*), and *Photorhabdus* (*Enterobacteriaceae*), carry *lux* operon genes. The luminescence operons of some of these bacteria also contain genes involved in the synthesis of riboflavin, *ribEBHA*, and in some species, regulatory genes *luxI* and *luxR* are associated with the *lux* operon. In well-studied cases, *lux* genes are coordinately expressed in a population density-responsive, self-inducing manner called quorum sensing. The evolutionary origins and physiological function of bioluminescence in bacteria are not well understood but are thought to relate to utilization of oxygen as a substrate in the luminescence reaction.

Keywords Bioluminescence · Bacterial luciferase · *Aliivibrio* · *Photobacterium* · *Vibrio* · *lux* genes

Abbreviations

acyl-HSL	Acyl-homoserine lactone
$FMNH_2$	Reduced flavin mononucleotide
Kb	Kilobases (thousand nucleotides)
kD	Kilodaltons

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Mb	Megabases (million nucleotides)
RCHO	Long-chain fatty aldehyde
RCOOH	Long-chain fatty acid

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1 Introduction

Light production by bacteria is one of several evolutionarily distinct kinds of bioluminescence, other kinds of which are found in various terrestrial and marine eukaryotic organisms [76, 199]. Bacterial bioluminescence has been known since 1875, when Pflüger [152] correlated the presence of bacteria in the surface slime of a fish with luminescence [74, 159]; earlier observations of luminescence, during the 1700 and 1800s, from various sources were likely due similarly to the presence of luminous bacteria, such as saprophytes or parasites [72, 73]. The oxygen-dependence of bacterial luminescence, first revealed by Boyle [24], who showed that light produced by decaying fish required air, suggests light production in bacteria arose evolutionarily after oxygen levels began to rise through the activities of early cyanobacteria, oxygenic phototrophs, approximately 2.4 billion years ago.

All luminous bacteria, as far as known to date, utilize the same enzymatic reaction for light production, based on bacterial luciferase. Phylogenetic analysis suggests that the genes coding for the bacterial luminescence enzymes arose once evolutionarily. Currently, luminous bacteria are grouped primarily in one *Gammaproteobacteria* family, *Vibrionaceae*. Some luminous members of closely related families exist, however, having apparently acquired the genes for luminescence by horizontal gene transfer. Historical perspectives on the first isolations and analyses and early taxonomy of luminous bacteria link the study of these bacteria to the origins of general microbiology [44, 57, 73, 159]. This chapter

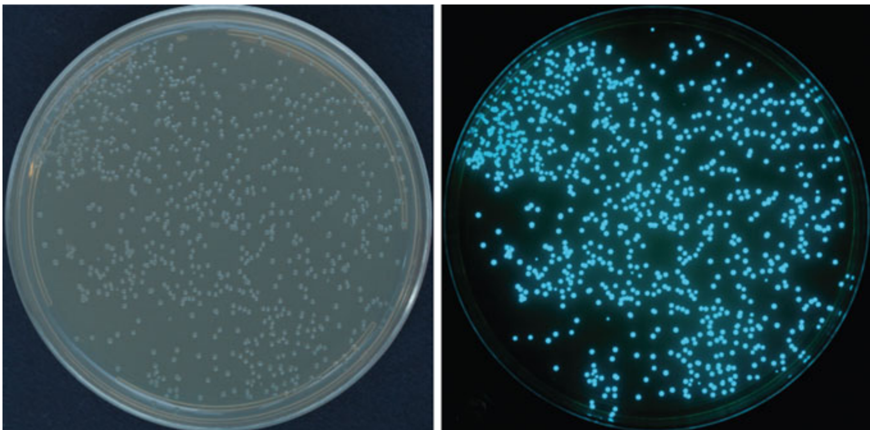

Fig. 1 Bacterial luminescence. Colonies of *P. mandapamensis* from the light organ of the cardinalfish *Siphamia tubifer* (Perciformes: Apogonidae) are shown growing on a nutrient seawater agar plate. The plate was photographed in room light (*left*) and (the same plate) in the dark by the light produced by the bacteria (*right*). From Dunlap et al. [46]

outlines the current systematics of luminous bacteria, provides an overview of the biochemistry and genetics of bacterial luminescence, and concludes with a discussion of the evolutionary origin and function of bacterial luminescence.

2 Species of Luminous Bacteria

Thirty or more species of bacteria have strains that make light visible to the eye (Fig. 1) or that at least carry genes for luminescence, the *lux* genes (Table 1). These bacteria, which are all Gram-negative, group phylogenetically as members of three families of *Gammaproteobacteria*: *Vibrionaceae* (*Aliivibrio*, *Photobacterium*, *Vibrio*), *Enterobacteriaceae* (*Photorhabdus*), and *Shewanellaceae* (*Shewanella*). It should be noted, however, that most *Vibrionaceae* species are not luminous and apparently lack *lux* genes and that only the few listed species in *Enterobacteriaceae* and *Shewanellaceae* are known to be luminous. Also, many of the species listed have nonluminous strains that may or may not lack *lux* genes [4, 19, 38, 60, 204]. The low number of luminous species, relative to the many described species in *Vibrionaceae*, is consistent with loss of the *lux* genes over evolutionary time from the ancestors of many lineages within the family. Furthermore, nonluminous variants apparently can arise readily through the loss of one of more core genes of the *lux* operon, *luxCDABEG* (e.g., [204]). It should be noted here also that several species previously classified variously as members of *Photobacterium* or *Vibrio* (i.e., *fischeri*, *salmonicida*, *logei*, and *wodanis*) have been reclassified as members of

Table 1 Species and ecological sources of luminous bacteria

Species	Sources	Selected references
Marine		
Aliivibrio		
fischeri	Coastal seawater, light organs of squid and fish	[22, 59, 157, 161, 163, 164, 188]
logei	Coastal seawater, sediment	[9, 12, 188]
salmonicida	Tissue lesions of Atlantic salmon	[91, 139, 188]
sifiae	Coastal seawater	[9, 209]
"*thorii*"	Light organs of squid	[9, 54]
wodanis	Coastal seawater, diseased farmed salmon, light organs of squid	[9, 115, 188]
Photobacterium		
aquimaris	Coastal seawater	[208]
damselae	Coastal seawater	[173, 189]
kishitanii	Light organs and skin of fish	[6, 7]
leiognathi	Coastal seawater, light organs of fish	[23, 45, 65, 158]
mandapamensis	Coastal seawater, light organs of fish	[85, 93, 157, 191]
phosphoreum	Coastal and pelagic seawater	[6, 7, 25, 202]
Candidatus Photodesmus		
katoptron	Light organs of *Anomalops katoptron*	[80, 86–88, 203]
blepharon	Light organs of *Photobelpharon palpebratus*	[80, 87, 88, 203]
Shewanella		
hanedai	Seawater and sediment	[92]
woodyi	Seawater and squid ink	[117]
Vibrio		
azureus	Coastal seawater	[207]
campbellii	Coastal seawater	[107, 175]
chagasii	Coastal seawater, surfaces and intestines of marine animals	[182, 189]
harveyi	Coastal seawater, sediment	[69, 142, 157, 163, 206]
jascicida	Coastal seawater	[57, 193]; H. Urbanczyk (pers. comm.)
mediterranea[a]	Coastal seawater	[153]
orientalis	Seawater, surface of shrimp	[205]
owensii	Coastal seawater	[193]; H. Urbanczyk (pers. comm.)
sagamiensis	Coastal seawater	[210]
splendidus	Coastal seawater	[20, 138]
vulnificus	Coastal seawater, oysters	[147, 189]
Brackish/Estuarine		
Vibrio		
cholerae	Estuaries, bays, coastal seawater	[93, 148, 154, 215]
Terrestrial		

(continued)

Table 1 (continued)

Species	Sources	Selected references
Photorhabdus		
asymbiotica	Human skin lesions	[53, 58, 99, 149, 200]
luminescens	Insect larvae infected with heterorhabditid nematodes	[21, 36, 58, 181]
temperata	Insect larvae infected with heterorhabditid nematodes	[58, 181]

[a] Ability of this species to luminescence has not been confirmed; the single strain reported as luminous [153] might not be available

Aliivibrio, a change that resolves long-standing confusion on the evolutionary relationships of these bacteria with members of *Vibrio* and *Photobacterium* [9, 188].

The majority of the luminous bacteria, members of *Aliivibrio*, *Photobacterium*, *Vibrio*, and *Shewanella*, are found in the marine environment. Luminous trains of *Vibrio cholerae* can be isolated from brackish environments and freshwater as well as from coastal seawater. Depending on the species, these bacteria occur free in seawater and in sediments or more commonly are associated with surfaces and gut tracts of marine animals as saprophytes and commensal symbionts (e.g., [160, 190]). They also occur as parasites of marine animals and as highly specific mutualistic bioluminescent light organ symbionts of many marine fish and squid. In contrast, *Photorhabdus* species occur in terrestrial environments and as symbionts of terrestrial heterorhabditid nematodes [195]. Several of the species listed in Table 1 were described recently, and others have been recognized only recently as luminous or as carrying *lux* genes. This progress suggests that future studies, especially those employing whole genome sequence analysis (e.g., [193]), will identify many more species and strains of bacteria that are luminous or at least carry *lux* genes.

Most luminous bacteria are culturable on laboratory media. Some, however, have not yet been brought into culture despite many attempts [81]. These not-yet cultured luminous bacteria are symbiotic in the subocular light organs of flashlight fish (Anomalopidae) and the escal light organs of members of many families of deep-sea anglerfish (order Lophiiformes). The inability to culture these bacteria suggests they are obligately dependent on their host fish for nutrients necessary for reproduction or conditions supporting their survival [81]. Early phylogenetic work placed these bacteria in *Vibrionaceae* and as a species distinct from the luminous bacteria known at the time [80, 81, 83, 84]. Recent analyses of the bacteria from light organs of two species of anomalopid fish, using multilocus phylogenetic analysis and whole genome sequence analysis, have identified the bacteria as members of a new *Vibrionaceae* genus, *Candidatus* Photodesmus, with two new species, *Candidatus* Photodesmus katoptron and *Candidatus* Photodesmus blepharon, from *Anomalops katoptron* and *Photoblepharon palpebratus*, respectively. The genomes of *Ca*. Photodesmus katoptron and *Ca*. Photodesmus blepharon, approximately 1.0 and

1.1 Mb, respectively, are massively reduced compared to the genomes of other members of *Vibrionacaeae*, which typically are 4.5–6.0 Mb, with many metabolic and regulatory genes lost. Genome reduction and gene loss apparently account for the inability of these bacteria to grow in laboratory culture [86–88]. For the deep-sea anglerfish, no additional information is available at this time on the species-level phylogenetic placement of their symbiotic luminous bacteria.

Despite much recent progress in clarifying the taxonomy of luminous bacteria (Table 1), the ecological incidence and species-level diversity of luminous bacteria remain incompletely defined. One problem is that the luminescence phenotype can be lost; strains luminous on primary isolation often become dim or dark in laboratory culture [3, 134, 171]. Another is that some species that grow well in laboratory culture at room temperature, i.e., *Aliivibrio logei* and *Shewanella hanedai*, typically produce light visible to the eye only when grown at cooler temperatures. In some cases, i.e., luminous bacteria infecting crustaceans; [67] and strains of *Aliivibrio fischeri* symbiotic with the Hawaiian sepiolid squid, *Euprymna scolopes* [22], the bacteria produce a high level of light in their natural habitat but produce little or no light when grown in laboratory culture. A further complication is that strains of some bacteria, such *V. cholerae*, are known to carry *lux* genes but apparently do not express them (e.g., [93, 148, 154, 215]). In addition, bacteria identified as related to *Vibrio harveyi* and *Vibrio cincinnatiensis* carry the *lux* genes but have been found to have *lux* gene mutations that result in a dark phenotype [143]. Although the phylogenetically scattered incidence of bacteria with *lux* genes in *Vibrionaceae* presumably relates to different ecologies of the different species, it is not obvious how having and expressing *lux* genes contributes to the lifestyle of most luminous bacteria; there are no obvious ecological differences between luminous and nonluminous species except in the case of those species that are bioluminescent symbionts of bacterially luminous fish and squid. On-going and future studies that examine the incidence, *lux* gene content, and phylogenetic placement of luminous bacteria will undoubtedly expand understanding of the species diversity and ecology of bacteria able to produce light.

3 Biochemistry of Bacterial Luminescence

Light emission in bacteria is catalyzed by a uniquely bacterial kind of luciferase, a heterodimeric protein of approximately 80 kD, composed of α (40 kDa) and β (37 kDa) subunits, with homology to long-chain alkane monooxygenases [76, 104]. The enzyme mediates the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain aliphatic (fatty) aldehyde (RCHO) by O_2 to produce blue-green light according to the following reaction.

$$\mathbf{FMNH_2 + O_2 + RCHO \overset{\textit{luciferase}}{\Rightarrow} FMN + H_2O + RCOOH + light\,(490\,nm)}$$

Along with bacterial luciferase, the substrates, $FMNH_2$ and long-chain fatty aldehyde, are specific to the bacterial luminescence reaction; bioluminescent eukaryotes employ different chemistries and luciferases that are not homologous at the protein or gene sequence levels to bacterial luciferase [76]. In the luminescence reaction, binding of $FMNH_2$ by the enzyme is followed by interaction with O_2 to form a flavin-4a-hydroperoxide. Association of this complex with aldehyde forms a highly stable intermediate, the slow decay of which results in oxidation of the $FMNH_2$ and aldehyde substrates and the emission of light [76, 79]. Quantum yield for the reaction has been estimated at 0.1–0.2 photons. The reaction is highly specific for $FMNH_2$, and the aldehyde substrate in vivo is likely to be tetradecanal. $FMNH_2$ is provided by the activity of an NADH:FMN oxidoreductase (flavin reductase), which taps reductant from NADH generated in cellular metabolism, for example, glycolysis and the citric acid cycle. Transfer of reductant from $FMNH_2$ to luciferase occurs by free diffusion. Synthesis of the long-chain aldehyde is catalyzed by a fatty-acid reductase complex composed of three polypeptides, an NADPH-dependent acyl protein reductase (r, 54 kDa), an acyl transferase (t, 33 kDa), and an ATP-dependent synthetase (s, 42 kDa). The complex has a stoichiometry of $r_4s_4t_{2–4}$, and its activity is essential for the production of light in the absence of exogenously added aldehyde [79, 121, 183, 201]. Luciferases from different species of luminous bacteria exhibit substantial amino acid residue and nucleotide sequence identity [45, 122], consistent with a common evolutionary origin of luminescence in bacteria.

4 Bacterial *lux* Genes

The *lux* operon, *luxCDABEG*, contains the genes necessary for light production in bacteria (Fig. 2). The *luxA* and *luxB* genes code for the α and β subunits of bacterial luciferase, respectively, *luxC*, *luxD*, and *luxE* genes, respectively, code for the r, s, and t polypeptides of the fatty-acid reductase complex that synthesizes and recycles aldehyde substrate for luciferase, and *luxG*, codes for flavin reductase [112, 122, 141, 178, 183]. The absence of *luxG* from the *lux* operons of *Photorhabdus luminescens* and newly characterized species of *Ca.* Photodesmus (Fig. 2) apparently is compensated for by the activity of a flavin reductase activity coded for by an *Escherichia coli fre*-like gene. Homologues of the *fre*-like gene are found in various luminous bacteria [212–214].

An additional gene, *luxF*, which codes for a nonfluorescent flavoprotein, is present in the *lux* operons of *Photobacterium*, between *luxB* and *luxE* (Fig. 2). The LuxF protein might function in the luminescence system by scavenging an inhibitory side product of the luciferase reaction [130], but it is not necessary for light production, even in those *Photobacterium* species that normally carry this gene [93]. The *luxF* gene apparently has been secondarily lost in *Photobacterium leiognathi* [5].

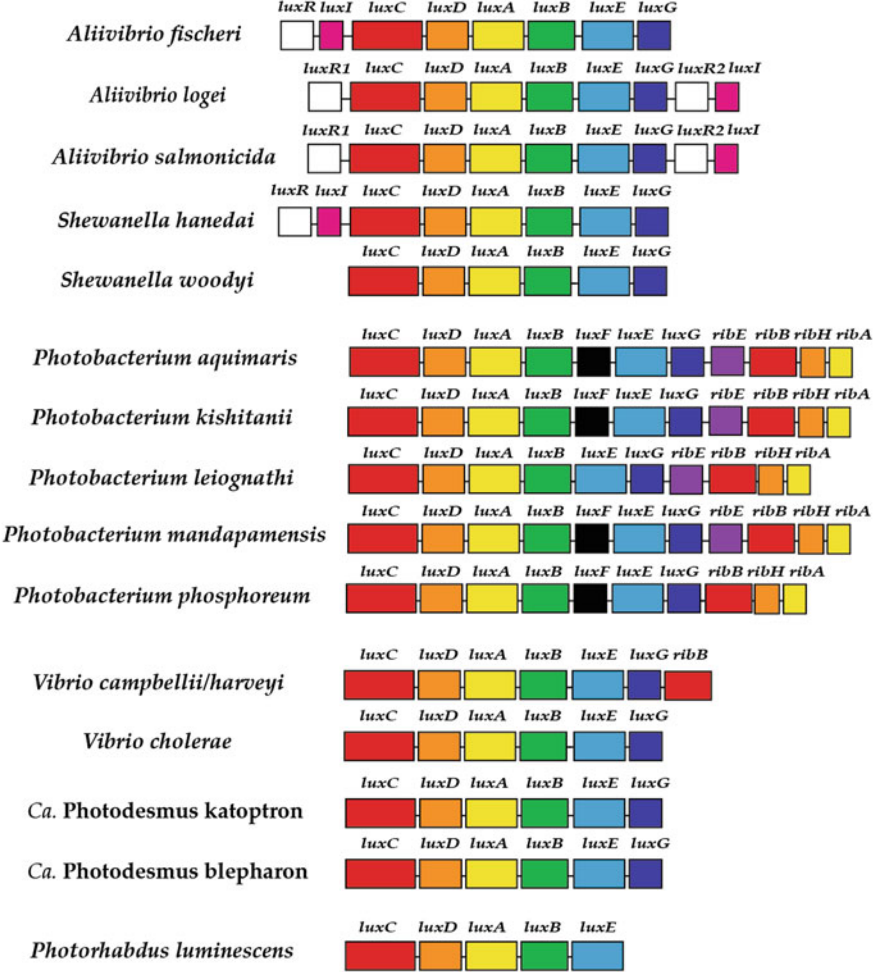

Fig. 2 Bacterial luminescence (*lux*) genes. Shown are the gene content and gene order of *lux* operons for those bacteria for which complete *lux* operon sequence data are available. Contiguous genes of the *lux* operons are aligned to highlight commonalities and differences. Four distinct types of *lux* operons are evident based on commonalities of gene content, organization, and sequence similarity: (1) *Aliivibrio/Shewanella* type, with *luxI/luxR* regulatory genes; (2) *Photobacterium* type, with *ribEBHA* genes forming a *lux-rib* operon; (3) *Vibrio/Candidatus* Photodesmus type, without linked regulatory genes; and (4) *Photorhabdus* type, composed of just five core *lux* genes, *luxCDABE*. Additional species of luminous bacteria are listed in Table 1. See text for details and for information on accessory genes

In *Photobacterium*, genes involved in the synthesis of riboflavin, *ribEBHA*, are part of the *lux* operon, forming a *lux-rib* operon, *luxCDABFEG-ribEBHA* (Fig. 2; [8, 100, 101, 113, 176]). The absence of a transcriptional stop or other regulatory site between the *lux* and *rib* genes indicates that these genes are coordinately

expressed from a single promoter upstream of *luxC*. Strains of *P. phosphoreum* lack *ribE*, which presumably was lost in the divergence from an ancestral *Photobacterium* that gave rise to this species. The presence of genes for synthesis of riboflavin as part of the *lux* operon might enhance light production by ensuring coordinate synthesis of luciferase and substrates for the enzyme. The *lux* operon of *V. campbellii* (previously classified as *V. harveyi*; [107]) contains *ribB*, coding for 3,4-dihydroxy-2-butanone 4-phosphate synthase, a key enzyme in riboflavin synthesis (referred to originally as *luxH*; [179]). In *A. fischeri*, although *ribB* is not part of the *lux* operon, its expression nonetheless is under the same regulatory control as the *lux* genes [26].

The isolation of a luminous strain of *V. campbellii* was first reported several years ago [175]. More recently, certain strains identified as *V. harveyi*, for example, BAA-1116 (a.k.a. BB120, which has been used extensively in studies of quorum sensing, e.g., [15]), were identified by whole genome sequence analysis as members of *V. campbellii* [107]. Careful phylogenetic analysis of strains currently referred to as *V. harveyi* is therefore needed to confirm their identity and the *lux* operon gene organization of this species.

In *Photobacterium*, many strains of *P. leiognathi* carry two intact and apparently functional *lux-rib* operons in their genomes [8]. The two operons, *lux-rib*$_1$ and *lux-rib*$_2$, are distinct in sequence and genomic location. Phylogenetic analysis indicates that *lux-rib*$_1$ and *lux-rib*$_2$ are more closely related to each other than either is to the *lux* and *rib* genes of other bacterial species [8]. These findings indicate that *lux-rib*$_2$ did not arise by a gene duplication event, and they exclude interspecies horizontal transfer as the origin of *lux-rib*$_2$ in *P. leiognathi*; instead, the second operon apparently was acquired by horizontal gene transfer from a lineage of *P. leiognathi* that either has gone extinct or has not yet been sampled. The *P. leiognathi lux-rib*$_2$ operon has also been found in two strains of *P. mandapamensis*, which also carry a normal *P. mandapamensis lux-rib* operon, and in a strain of *P. damselae*, a species not previously known to be luminous [189]. In *Photobacterium aquimaris* [208], the *lux* operon of one of two luminous strains apparently has been replaced by horizontal acquisition of the *lux* operon of *Photobacterium mandapamensis* [192].

In addition to the presence of the *ribEBHA* genes as part of the *lux* operon of *Photobacterium*, other genus-specific differences are evident in the genes upstream of and flanking the *lux* operons of luminous bacteria (Fig. 2). In *P. mandapamensis*, the *lux-rib* operon is preceded by *lumQ* and *lumP*, which form the lumazine operon. The function of *lumQ* is not yet known, although it is thought to code for a DNA-binding protein [109]. LumP, a 21-kDa fluorescent accessory protein referred to as lumazine protein, functions to enhance the intensity of light emission and to shift the emission wavelength of luciferase from blue-green (495 nm) to blue (475–486 nm) [102, 145, 146, 151]. A *lumP* gene is present just upstream of *luxC* in *Photobacterium phosphoreum* and *Photobacterium aquimaris*, but *lumQ* is absent [192]. The LumP protein, which has been isolated from *P. phosphoreum* and from a strain of *P. mandapamensis* (previously classified as a strain of *P. leiognathi*) and also purified from *P. kishitanii*, contains a noncovalently bound

fluorophore, 6,7-dimethyl-8-ribityllumazine, the immediate biosynthetic precursor of riboflavin [145, 165, 172]. In *P. leiognathi lumP* is not found, although approximately 200 nucleotides of the *P. leiognathi luxC-lumQ* intergenic region can be aligned to the *P. mandapamensis lumP* gene [8]. The activity of the LumP protein apparently accounts for the blue-shifted luminescence of *P. mandapamensis* compared to *P. leiognathi*, one of the diagnostic traits distinguishing these two species [5, 102, 145, 146, 151, 93]. The genes flanking the *P. leiognathi* and *P. mandapamensis lux-rib* operons are homologous to a single contiguous region in nonluminous *P. angustum* [8, 108–111, 113].

In the examined *Aliivibrio* species, regulatory genes, *luxI* and *luxR*, which control transcription of the *lux* operon (described below), are upstream of or flank the *luxCDABEG* genes (Fig. 2). The *luxI* gene codes for an acyl–homoserine lactone (acyl-HSL) synthase [166], and *luxR* codes for a receptor protein that interacts with acyl-HSL to activate transcription of the *lux* operon [52]. In *Aliivibrio fischeri*, *luxI* is the first gene of the *lux* operon, and *luxR*, upstream of *luxI*, is divergently transcribed (Fig. 2). The *lux* operon of *Shewanella hanedai* has the same gene arrangement. This similarity, together with a high degree of *lux* gene sequence identity suggests that *S. hanedai* acquired its *lux* operon by horizontal transfer from *A. fischeri* or the ancestor of *A. fischeri* [189]. In *Aliivibrio salmonicida*, a bacterium that requires exogenous addition of aldehyde to produce a high level of light [55], two *luxR* genes, homologous to *A. fischeri luxR*, flank the *lux* operon; a *luxI* gene also is present, divergently transcribed from the downstream *luxR* (Fig. 2; [91, 139]). The same arrangement of *luxI* and *luxR* genes as in *A. salmonicida* is present in *Aliivibrio logei* [119]. In contrast to *A. salmonicida*, however, exogenous addition of aldehyde is not required for a high level of light production in *A. logei* [119]; mutations in *luxD* account for the exogenous aldehyde requirement of *A. salmonicida*. Genes flanking the *lux* operons of other luminous *Aliivibrio* species (Table 1) apparently have not yet been characterized. An accessory protein, yellow fluorescent protein (YFP), is present in *Aliivibrio sifiae* (previously referred to as *A. fischeri*; [9, 209] and shifts the emission wavelength of luminescence toward yellow [11, 33, 150, 162]; the YFP gene apparently is not linked to the *lux* operon.

With respect to *S. hanedai* and *S. woodyi*, comparison of genes flanking the *lux* operons suggested that these species had acquired *lux* genes from a member of *Aliivibrio* [96], a possibility confirmed through phylogenetic analysis [189]. In *Photorhabdus* species, the *luxCDABE* genes also might have been acquired by horizontal gene transfer, possibly from an ancestor of *V. harveyi* [61, 121, 123]. Phylogenetic analysis of the *Photorhabdus lux* genes, however, neither supports nor contradicts horizontal acquisition of the *lux* genes by *Ph. luminescens* [189]. Certain instances of horizontal acquisition of *lux* genes have also been found for *Vibrio* [189]. The one known luminous strain of *Vibrio vulnificus*, the human pathogen [147], apparently acquired its *lux* genes from *V. harveyi*, and in *V. chagasii*, a species not described as luminous [182], two luminous strains were identified and apparently had acquired their *lux* genes from *V. harveyi* and *V. splendidus*, respectively [189].

The arrangement of genes associated with *luxCDABEG* of the examined *Vibrio* species differs substantially from that in *Photobacterium* and in *Aliivibrio* (Fig. 2). Regulatory genes controlling transcription of the *lux* operon are not part of and are not adjacent to the *lux* operons of those *Vibrio* species examined; specifically, a *luxR* gene, referred to here as $luxR_{\mathrm{Vh}}$ which is not homologous to *A. fischeri luxR*, is not physically associated with the *lux* operon in *V. campbellii* (*V. harveyi*). Also, with the exception of *ribB* in *V. campbellii*, genes involved in riboflavin synthesis in *Photobacterium*, *ribEBHA*, are not part of the *lux* operon in *Vibrio*. Conservation of *luxCDABEG* as a unit might reflect a need for close interaction of luciferase with the enzymes, fatty acid reductase and flavin reductase, producing substrates for the reaction, for efficient light production. However, it is not obvious what led to the genus-specific *lux* operon gene content and organization in *Aliivibrio*, *Photobacterium*, and *Vibrio* (Fig. 2), three closely related genera of *Vibrionaceae*.

5 Regulation of *lux* Operon Expression

The production of light consumes a substantial amount of energy, through the synthesis and activity of the Lux proteins [41]. The retention and expression of *lux* genes in many bacteria, despite this energetic cost, therefore indicates that the activity of the luminescence system must benefit these bacteria, physiologically or ecologically, as discussed below. Furthermore, this energetic cost presumably accounts for the population density-responsive regulation of *lux* operon expression characteristic of many luminous bacteria. Originally called autoinduction and discovered through study of the pattern of luminescence and luciferase synthesis of *V. harveyi* in batch culture [132, 136], this gene regulatory mechanism is now referred to as quorum sensing to reflect its relationship with population density [66, 70, 77, 124]. At low population density, very little luciferase is synthesized, and consequently little light is produced, whereas at high population density, luciferase levels are induced 100–1,000-fold and light levels increase by 10^3–10^6-fold. This population density-responsive induction of luciferase synthesis and luminescence is controlled in part by the production and accumulation in the cell's local environment of small secondary metabolite signal molecules, called autoinducers, which function via regulatory proteins to activate or derepress transcription of the *lux* operon. Quorum sensing has been studied intensively in two luminous bacteria, *V. harveyi* and *A. fischeri*. With respect to *V. harveyi*, a strain used extensively in quorum-sensing research, BAA-1116, also known as BB120 (e.g., [15]), recently was recognized through whole genome sequence analysis to be a member of *V. campbellii* [107]. The information related below is therefore provisionally ascribed to *V. campbellii*, pending resolution of the taxonomic status of strains called *V. harveyi* that have been used in the various studies of quorum sensing. The quorum-sensing systems of these two bacteria, *V. campbellii* and *A. fischeri*, briefly outlined below, differ substantially at genetic and chemical levels. Despite the many

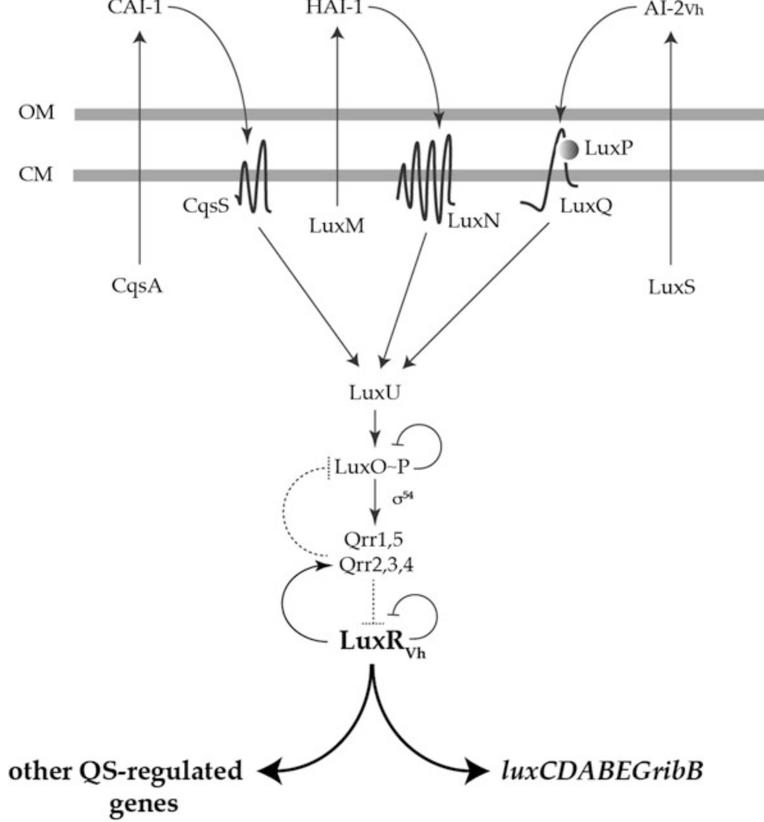

Fig. 3 Quorum-sensing regulatory circuitry in *Vibrio campbellii* (*Vibrio harveyi*). The expression of *lux* operon, and of other quorum-sensing–regulated genes, in *V. campbellii* (previously classified as *V. harveyi*; [107] is coordinated by three chemically distinct autoinducers, HAI-1, AI-2_{Vh}, and CAI-1, that modulate the phosphorylation state of *luxU*. The synthesis of each autoinducer is catalyzed by a different protein, LuxM, LuxS, and CqsA, and each is recognized by a different cytoplasmic membrane-associated two-component histidine–kinase receptor, LuxN, LuxPQ, and CqsS, respectively. Low concentrations of the autoinducers lead to phosphorylation of LuxO and via quorum–regulatory RNAs to the destabilization of the *luxR*$_{Vh}$ transcript, thereby blocking *lux* operon transcriptional activation by $LuxR_{Vh}$. High concentrations of the autoinducers reverse the phosphorylation cascade, allowing formation of $LuxR_{Vh}$ and activation of *lux* operon transcription. Arrows indicate positive interactions or transcriptional activation, whereas bars indicate negative interactions or blocking of transcription. See the text for details and references. Redrawn from [185]

differences, however, several commonalities to the two systems have been identified [44]. Recent publications provide additional details on quorum sensing in these bacteria as well as in nonluminous bacteria [14, 44, 155].

In *V. campbellii*, *lux* operon expression is controlled by a multicomponent phosphorylation/dephosphorylation cascade (Fig. 3). Three chemically distinct autoinducers are involved: 3-hydroxybutanoyl-HSL (harveyi autoinducer-1,

HAI-1), (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate (*V. harveyi* autoinducer-2, AI-2_{Vh}), and (S)-3-hydroxytridecan-4-one (cholerae autoinducer, CAI-1) [27, 31, 90]. Synthesis of HAI-1 is dependent on LuxM [16], synthesis of AI-2_{Vh} is catalyzed by LuxS [167], and synthesis of CAI-1 is catalyzed by CqsA [97, 198]. Each of these molecules is recognized by a different cytoplasmic membrane-associated two-component histidine-kinase receptor, LuxN [16, 64], LuxPQ [18], and CqsS [89], respectively. When concentrations of the autoinducers are low, such as at low population density or in habitats in which the autoinducers diffuse away rapidly from cells (i.e., in seawater), the receptor proteins function as kinases, transferring phosphate to LuxU, a histidine-phosphotransfer protein. LuxU then transfers the phosphate to LuxO, a DNA-binding response regulator, the expression of which is subject to repression by LuxT [13, 17, 27, 62, 63, 106, 114, 126, 177, 197]. LuxO $\sim$ P, together with sigma factor σ^{54}, then activates the expression of genes coding for five small quorum-regulatory RNAs (Qrr), Qrr1 through Qrr5 [103, 184]. The Qrr RNAs bind and destabilize the *luxR*$_{Vh}$ transcript, blocking production of LuxR$_{Vh}$ protein, the transcriptional activator of the *lux* operon [170, 180], and thereby preventing activation of *lux* operon transcription. Conversely, once autoinducer concentrations have attained high levels in the cell's local environment, they bind to their receptors, causing the receptors to switch from kinases to phosphatases, leading to the dephosphorylation of LuxO. With the resulting cessation of *qrr* gene transcription, a *luxR*$_{Vh}$ message is produced and translated, and LuxR$_{Vh}$ activates *lux* operon transcription. Negative autoregulation of LuxR$_{Vh}$ adds additional complexity to this system [28, 125], as does the negative autoregulation of LuxO, posttranscriptional control of LuxO by Qrr sRNAs [185], and involvement of 3′:5′-cyclic AMP (cAMP) and camp receptor protein (CRP) [29, 30, 34, 127, 137, 187]. The complexity of this regulatory system apparently benefits *V. campbellii* by allowing a fine-tuning of its quorum-sensing response to differences in the various habitats the bacterium colonizes [140, 185, 196].

In *A. fischeri*, quorum-sensing control of luminescence in *A. fischeri* involves a population-density–dependent accumulation of the autoinducer 3-oxo-hexanoyl-homoserine lactone (AI-1), a membrane-permeant molecule that triggers induction of *lux* operon transcription when AI-1 reaches a critical concentration (Fig. 4). Synthesis of AI-1 is catalyzed by LuxI, an acyl–homoserine lactone synthase, from S-adenosyl methionine and acyl-HSL. The regulatory genes, *luxR*$_{Af}$ and *luxI*, are directly linked to the *lux* operon. The *luxR*$_{Af}$ gene, which is upstream of the *lux* operon and divergently transcribed from it, encodes a transcriptional activator protein, LuxR$_{Af}$, which associates with AI-1, forms a complex that binds at a site in the *lux* operon promoter and facilitates the binding of RNA polymerase, thereby activating transcription of the genes for light production, *luxICDABEG*. Because *luxI* is a gene of the *lux* operon, increased transcription leads to increased production of LuxI protein and increased synthesis of AI-1, in an autocatalytic, positive feedback manner. The result is a rapid and strong induction of luciferase synthesis and luminescence [48, 50–52, 94, 166, 174].

Several other regulatory factors modulate quorum-sensing in *A. fischeri*. These factors include: cAMP and CRP, which activate transcription of *luxR*$_{Af}$ and

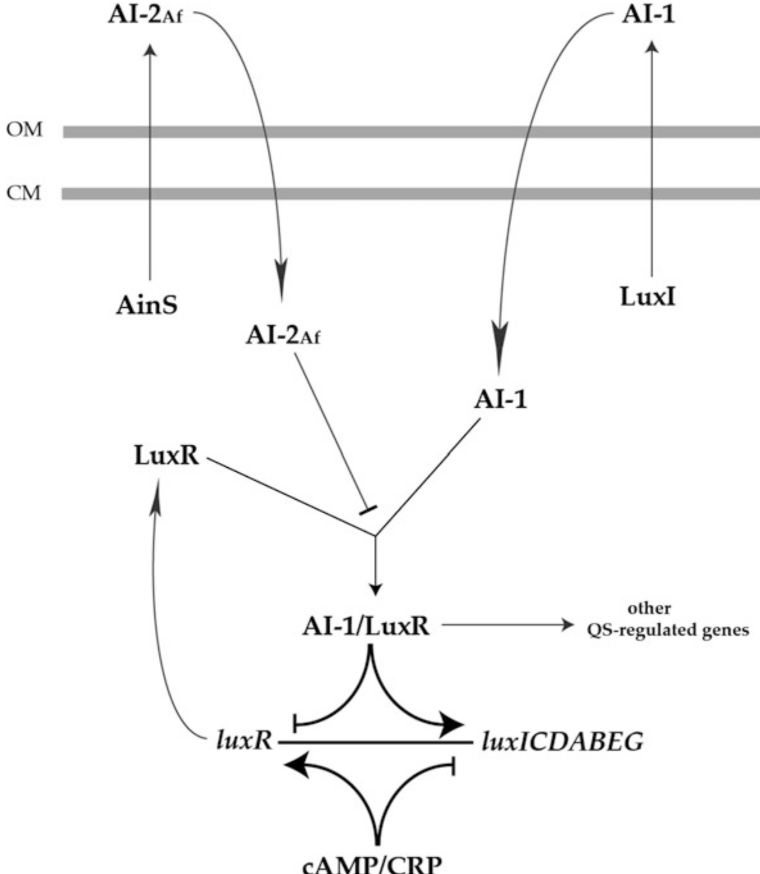

Fig. 4 Quorum-sensing control of luminescence in *A. fischeri*. The expression of the *lux* operon, and of other quorum-sensing-regulated genes, in *A. fischeri* is mediated primarily by the concentration of AI-1, which forms a complex with $LuxR_{Af}$. Synthesis of AI-1 is dependent on LuxI, and the AI-1/$LuxR_{Af}$ complex activates *luxICDABEG* transcription. Together with cAMP, the CRP protein activates expression from the $luxR_{Af}$ promoter, increasing synthesis of $LuxR_{Af}$ and potentiating the system to be induced once sufficient AI-1 has accumulated. The presence of *luxI*, coding for AI-1 synthase, as part of the *lux* operon, leads to increased expression from the *lux* operon promoter, stimulating AI-1 synthesis in an autocatalytic, positive feedback manner; the result is a rapid and strong induction of luciferase synthesis once a threshold concentration of AI-1 is attained. A second autoinducer, AI-2_{Af} interacts with $LuxR_{Af}$, interfering with the interaction between AI-1 and $LuxR_{Af}$. The hypothesized AI-2_{Af}/$LuxR_{Af}$ complex is thought to be transcriptionally less effective and therefore to function to delay the onset of AI-1/$LuxR_{Af}$ activation of *luxICDABEG* transcription. See the text for details and references

thereby potentiate the cell's response to AI-1 while repressing transcription of *luxICDABEG*; negative autoregulation of $luxR_{Af}$ expression by $LuxR_{Af}$/AI-1; a second autoinducer, octanoyl-HSL (AI-2_{Af}), which is synthesized by AinS and that interacts with $LuxR_{Af}$ apparently interfering with AI-1 binding and thereby

delaying *lux* operon induction; involvement of GroEL in production of active $LuxR_{Af}$; a homologue of the *V. harveyi* LuxO, which functions in *A. fischeri* as a repressor of luminescence apparently in a *qrr*-dependent manner; LitR, a protein with substantial sequence similarity to $LuxR_{Af}$ and which positively regulates *lux* operon expression; and an involvement of Fnr and LexA in *lux* operon expression [1, 2, 35, 37, 39, 40, 42, 43, 49, 56, 68, 71, 98, 126, 128, 129, 131, 169, 186]. As in *V. campbellii*, the complexity of inputs into the quorum-sensing regulatory circuitry in *A. fischeri* indicates both a tight integration of luminescence into the lifestyle of the bacterium and the ability to modulate *lux* operon expression in response to a variety of conditions.

6 Origin and Function of Luminescence in Bacteria

The conserved gene content and gene order of the *lux* operon in bacteria, *luxCDABEG* (Fig. 2), and the high levels of *lux* gene and Lux protein amino acid sequence identities among luminous bacteria (e.g., [122]) indicate that all known bacterial *lux* operons derive from a single common ancestor. The congruence of phylogenies based on *lux* genes and other protein coding genes (and the 16S rRNA gene) [189] indicates that bacterial luminescence arose within the *Vibrionaceae* lineage and mostly likely in the ancestor that gave rise to *Aliivibrio*, *Photobacterium*, and *Vibrio*.

The homology of bacterial luciferase to long-chain alkane monooxygenases [104] suggests that the light-emitting enzyme arose from this family of proteins, even though none of the other enzymes in the family emit light [76]. With some light emission, bacteria luciferase might have evolved under ecological selection, according to the following scenario [168]. A flavoprotein catalyzing fatty acid α-oxidation reactions with low chemiluminescent quantum yields is postulated to have mutated under hypoxic conditions to accept $FMNH_2$ as the flavin cofactor, generating a fortuitously high fluorescence yield, termed "protobioluminescence," via the 4a-hydroxy-FMNH product [168]. This flavin dependent, aldehyde-oxidizing protoluciferase produced sufficient light, and with an appropriate emission spectrum, to be detected by phototactic organisms. Ingestion by visually cueing animals of particles colonized and made luminous by these early luminous bacteria presumably enhanced their reproduction by bringing them into the animal's nutrient-rich digestive system, ensuring the emitter's survival and thereby possibly leading to selection for more intense light output [199]. It is possible that early evolutionary steps leading to protoluciferase involved oxygen detoxification activity that permitted early anaerobic organisms to survive an increasingly aerobic environment [120, 156]. An alternative hypothesis for the evolution of bacterial luciferase, involvement in DNA repair [32], has been refuted [194].

A single gene was hypothesized to encode bacterial protoluciferase [144]. Although a single-subunit protoluciferase, monomer or dimer, presumably would have differed somewhat from the modern-day luciferase α-subunit and therefore

might have produced light, the inability of either of the extant α or β subunits alone to produce light in vitro or in vivo [105] argues against the single-gene hypothesis. Alternatively, bacterial protoluminescence may have arisen following a gene duplication event that is postulated to have created *luxB* from *luxA* [10, 122, 144]. Based on amino acid sequence identities, a tandem duplication of the ancestral *luxA* gene, followed by sequence divergence in the duplicated gene, is thought to have given rise to *luxB*, leading to the formation of the heterodimeric luciferase present in extant luminous bacteria. Similarly, a tandem duplication of *luxB* followed by loss of approximately 300 nucleotides coding for N-terminus amino acids is thought to have given rise to *luxF* in a luminescent ancestor of *Photobacterium*; this gene apparently was later secondarily lost in the lineage giving rise to *P. leiognathi* [5, 10, 122, 144].

The association of the fatty-acid reductase genes, *luxCDE*, with *luxA* might have predated the *luxA* to *luxB* gene duplication event. Alternatively, the presence of ERIC sequences flanking *luxA* and *luxB* in *Ph. luminescens* [123] might mark an insertion of the *luxAB* genes into the fatty aldehyde reductase operon during the evolution of the bacterial luminescence system. The origins and evolution of other luminescence genes are not well understood [144]. The evolution of the bacterial luminescence system also involved recruitment of regulatory and other genes to the *lux* operon in some species (Fig. 2).

The energetic cost of light production, involving expenditures of carbon, nitrogen, and ATP in the synthesis of Lux proteins and in their enzymatic activities [41] indicates that luminescence has physiological or ecological importance for those bacteria able to express it. In the absence of selection to retain the *lux* genes, this cost would lead to loss of function through mutation and gene loss, an evolutionary scenario that probably accounts for the scattered incidence of luminous strains and species in *Vibrionacaeae*. As outlined above, bacterial luminescence might have arisen evolutionarily as a means of coping with oxygen. Consistent with this possible function, luciferase, as an oxidase, might function as a secondary respiratory chain that is active when oxygen or iron levels are too low for the cytoplasmic membrane-associated, ATP-generating electron transport system to operate. This activity would allow cells expressing luciferase to reoxidize reduced coenzyme even when oxygen levels are low [75, 76, 78, 135]. Supporting this view, growth of cytochrome-deficient luminous bacteria is dependent on induction of luciferase, limitation for iron stimulates light production, low oxygen levels promote the luminescence of some luminous bacteria, and luciferase synthesis can be induced under anaerobic conditions [47, 82, 116, 118, 133]. As an alternative or supplement to the electron transport system, the activity of luciferase in reoxidizing reduced coenzyme could permit cells of luminous bacteria in low oxygen habitats, such as in animal gut tracts, to continue to transport and metabolize growth substrates, thereby continuing to gain energy through substrate-level phosphorylation. Furthermore and consistent with the ecological selection scenario above, light production presumably facilitates dissemination of luminous bacteria. The feeding of animals on luminous particles (decaying tissues, fecal pellets, and moribund animals infected by luminous bacteria), to which they are attracted,

would bring the bacteria into the animal's nutrient-rich gut tract for additional rounds of reproduction followed by dispersal [78, 135]. Recent evidence is supportive of this possibility [211]. Alternatively, the function of the bacterial *lux* system might be to generate a halotolerant flavodoxin, with light emission an incidental consequence [95]. Future studies might provide additional support for these and other proposed functions for luminescence, such as a physiological role for luciferase activity in bioluminescent symbioses with fish and squid. Proposed functions will be held to the standard of a demonstrated selective benefit for luminescence, either physiological or ecological.

7 Outlook

Substantial progress has been made in the past few years in defining the taxonomy and phylogenetic relationships of luminous bacteria and more fully characterizing the biochemistry and genetics of bacterial luminescence. Despite this progress, current understanding of the physiological function and ecological benefit of luminescence in bacteria remains limited. The long-standing question, "Why do bacteria make light?" remains essentially unanswered. A more detailed knowledge of the evolutionary origins and biochemical uniqueness of bacterial luciferase (e.g., [104]) and a more comprehensive understanding of the phylogenetic distribution of *lux* genes through whole-genome sequence analysis (e.g., [193]) in the context of the ecology of these bacteria (e.g., [211]) are likely to provide clues. Particularly insightful, however, will likely be detailed comparative physiological analysis of genetically defined mutants (e.g., [116]), an approach that addresses the core question and provides an experimental foundation for testing specific functional hypotheses.

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Part II
Applications of Bioluminescence in Environment and Security

Application of Enzyme Bioluminescence in Ecology

Elena Esimbekova, Valentina Kratasyuk and Osamu Shimomura

Abstract This review examines the general principles of bioluminescent enzymatic toxicity bioassays and describes the applications of these methods and the implementation in commercial biosensors. Bioluminescent enzyme system technology (BEST) has been proposed in the bacterial coupled enzyme system, wherein NADH:FMN-oxidoreductase-luciferase substitutes for living organisms. BEST was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for medical, environmental, and industrial applications. For widespread use of BEST, the multicomponent reagent "Enzymolum" has been developed, which contains the bacterial luciferase, NADH:FMN-oxidoreductase, and their substrates, co-immobilized in starch or gelatin gel. Enzymolum is the central part of Portable Laboratory for Toxicity Detection (PLTD), which consists of a biodetector module, a sampling module, a sample preparation module, and a reagent module. PLTD instantly signals chemical–biological hazards and allows us to detect a wide range of toxic substances. Enzymolum can be integrated as a biological module into the portable biodetector–biosensor originally constructed for personal use. Based on the example of Enzymolum and the algorithm for creating new enzyme biotests with tailored characteristics, a new approach was demonstrated in biotechnological design and construction. The examples of biotechnological design of various bioluminescent methods for ecological monitoring were provided. Possible applications of enzyme bioassays are seen in the examples for medical diagnostics, assessment of the effect of physical load on sportsmen, analysis of food additives, and in practical courses for higher educational institutions and schools. The advantages of enzymatic assays are their rapidity (the period of time required does not exceed 3–5 min), high

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sensitivity, simplicity and safety of procedure, and possibility of automation of ecological monitoring; the required luminometer is easily available.

Keywords Bioluminescence · Ecological monitoring · Enzymatic assay · Immobilization · Integral water toxicity · Luciferase

Abbreviations

ADH	Alcohol dehydrogenase
BEST	Bioluminescent enzyme system technology
FMN	Flavin mononucleotide
$FMN{\cdot}H_2$	Reduced flavin mononucleotide
L	Luciferase
L + R	Coupled enzyme system: NADH:FMN-oxidoreductase-luciferase
LI	Luciferase index
P	Induction period
PLTD	Portable laboratory for toxicity detection
R	NADH:FMN-oxidoreductase
RCHO	Long-chain aliphatic aldehyde
RCOOH	Corresponding fatty acid
TC	Toxicity coefficient

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1 Introduction

Present-day analytical methods based on bioluminescent reactions catalyzed by various luciferases cover a wide range of analytical tasks from ecological monitoring of the environment (integral bioassays) to molecular diagnostics of various diseases and infections (high-sensitivity and specific bioluminescent immunoassays and heteroduplex analyses). The prospective viability of the application of bioluminescent methods is explained by their advantages:

- Broad range: Feasibility of constructing assays for a variety of processes and substances.
- High sensitivity: Possibility of detecting a target substance at concentrations down to 10^{-12} to 10^{-15} mol L^{-1}.
- Rapidity of measurement: Bioluminescent reaction lasts milliseconds, so usually the analysis time varies within the length of period from 1 to 30 min.
- Feasibility of constructing assays for individual specific molecules.
- The ability to test a number of key physiological and biochemical processes, for example, key metabolites such as NADH, flavins, glucose-6-phosphate, and so on; activities of key enzymes such as NAD^+ or NADH-dependent dehydrogenases and trypsin, among others; consumption of unique energy substrate such as ATP; intracellular dynamics of Ca^{++} (a universal regulator); ribosomal synthesis of protein molecules; genome structure and functioning; and others.
- Relative simplicity of methods.
- Availability of reagents and bioluminometers (devices for measuring light emission intensity).

The era of bioluminescent analytical studies began with the use of luminous bacteria enzymes, and continues to play an important role in bioluminescent analytics. Three factors determine the prospective viability of luminous bacteria and their enzymes in assays.

First, researchers had enough bacterial luciferase because it was possible to obtain large amounts of biomass; luminous bacteria had long been the only cultivated luminous organisms [1]. Recently, it has become possible to create recombinant organisms by introducing the *lux* genes into bacteria, which has solved the problem of getting luciferase from other luminous organisms for analytic purposes [2]. During those years when only bacterial luciferase was available, more than 100 methods were developed using luminous bacteria and their enzymes. Some of them are presented in the book series *Methods in Enzymology* published in 1978 and 1986 [3, 4] and in the reviews by Kratasyuk and Gitelson [5], Girotti et al. [6], and Roda et al. [7].

Second, bioluminescent assays using bacterial luciferase were characterized by high specificity of enzyme–substrate interactions.

Finally, it is known that the energy supply of bioluminescence occurs through the general metabolic chains of cells [8, 9]. This makes it possible to use the chains coupling enzymes with bacterial luciferase or build such chains artificially so that

the concentration of most key metabolites (and antimetabolites) or activities of key enzymes can be measured on the basis of luminescence.

At present, bacterial coupled enzyme systems NAD(P)H:FMN-oxidoreductase-luciferase are actively used in bioluminescent assays for ecology, medicine, agriculture, and other areas [7, 10]. In Reaction 1, luciferase catalyzes the oxidation of long-chain aliphatic aldehydes involving reduced flavin mononucleotide; one of the products of this reaction is a quantum of light in the blue-green spectrum (Reaction 1). To provide luciferase with reduced flavin mononucleotide, the luciferase reaction is coupled with the reaction catalyzed by NAD(P)H:FMN-oxidoreductase (Reaction 2).

$$\mathrm{FMN}\cdot\mathrm{H_2}+\mathrm{RCHO}+\mathrm{O_2}\xrightarrow{\textit{Luciferase}\ (L)}\mathrm{FMN}+\mathrm{RCOOH}+\mathrm{H_2O}+\mathrm{h}\nu \tag{1}$$

$$\mathrm{NAD(P)H}+\mathrm{FMN}+\mathrm{H}^{+}\xrightarrow{NAD(P)H\,:\,FMN-oxidoreductase\ (R)}\mathrm{NAD(P)}^{+}+\mathrm{FMN}\cdot\mathrm{H_2}, \tag{2}$$

Methods based on using enzyme–substrate systems from luminous bacteria can be nominally divided into two groups: selective analysis methods and integrated assays such as bioluminescent toxicity assays.

Despite the diversity of bioluminescent methods used in analytical studies, bacterial luminescence has long been the only bioluminescent system used for the assessment of chemical pollution of various objects or total toxicity of the test samples. Historically, the application of bacterial luminescence began with the use of luminous bacteria. Many works have been published describing the application of luminous bacteria for ecological monitoring [5, 6]. These methods made it possible to determine environmental pollution by comparing the luminous intensity of bacteria in the control and in the analyzed sample. As opposed to other test objects such as paramecia, algae, crustaceans, and so on, the bioluminescent bioassay was faster (the time of analysis didn't exceed 20 min); however, as with other living organisms, the essential disadvantage of this method was low accuracy of measurement caused by the "petulance" of live luminous bacteria: failure to maintain the stable state of bacterial culture during measurements and storage. The bacteria reacted to the appearance of toxic substances either by decreasing or by increasing the luminous intensity, which often led to ambiguous interpretation of results. That's why only qualified staff could work with bacteria. Because of these shortcomings a bioassay based on luminous bacteria didn't show very good results in ecological laboratories. To overcome those difficulties it was decided to use the enzymes of luminous bacteria both in soluble and immobilized states.

Since 1990, a new trend in bioluminescent analysis has been developing: bioluminescent enzyme system technology (BEST) [11], where the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase substitutes for living organisms in bioluminescent enzymatic toxicity bioassays. In the presence of toxic agents, enzymes from luminous bacteria more closely reflect the toxicity of living organisms than do the use of chemical analysis and other current methods

that cannot solve the pressing problem of how to detect, identify, and measure the contents of the numerous chemical compounds that differ in their physicochemical and toxic characteristics. Moreover, BEST was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors for medical, environmental, and industrial applications.

The present work examines the general principles of bioluminescent enzymatic toxicity bioassays and describes the applications of these methods and their implementation for commercial biosensors.

2 Principles of Bioluminescent Enzymatic Bioassays

The main principle of bacterial luciferase-based analysis methods is detection of toxic properties in test substances and mixtures by their effect on bioluminescent enzymatic reactions [12–15].

Bioluminescent assays are based on the phenomenon of luciferase inhibition by the components of analyzed mixtures (Fig. 1). Many approaches and methods have been developed for design of in vitro bioluminescent assays, based on the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase (L + R), especially for ecological monitoring.

2.1 The Bioassays Based on the Bacterial Coupled Enzyme System: NADH:FMN-Oxidoreductase-Luciferase

Application of enzymatic bioassays based on the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase for ecological monitoring instead of living organisms such as intact luminous bacteria, daphnia, algae, and other organisms that can be legally used in Russia is justified by the fact that NADH:FMN-oxidoreductase as part of enzymatic bioassays is present in all living organisms. That is why good correlation is expected between the effect of toxic substances on living organisms and on the coupled enzyme system of luminous bacteria. Integrated luciferase-based biotesting methods are mainly used for continuous rapid environmental monitoring in industrial and agricultural regions, for control over pollutant emissions of enterprises, for estimation of the efficiency of wastewater detoxification and of the control of water and wastewater treatment facilities, as well as for ecological risk assessment.

Bioluminescent integrated assays demonstrate changes in the bioluminescent signal as a reaction to the appearance of large-scale classes of polluting substances in the analyzed media. But the development of such assays depends on the influence of pollutants and xenobiotics on bioluminescence [16–18].

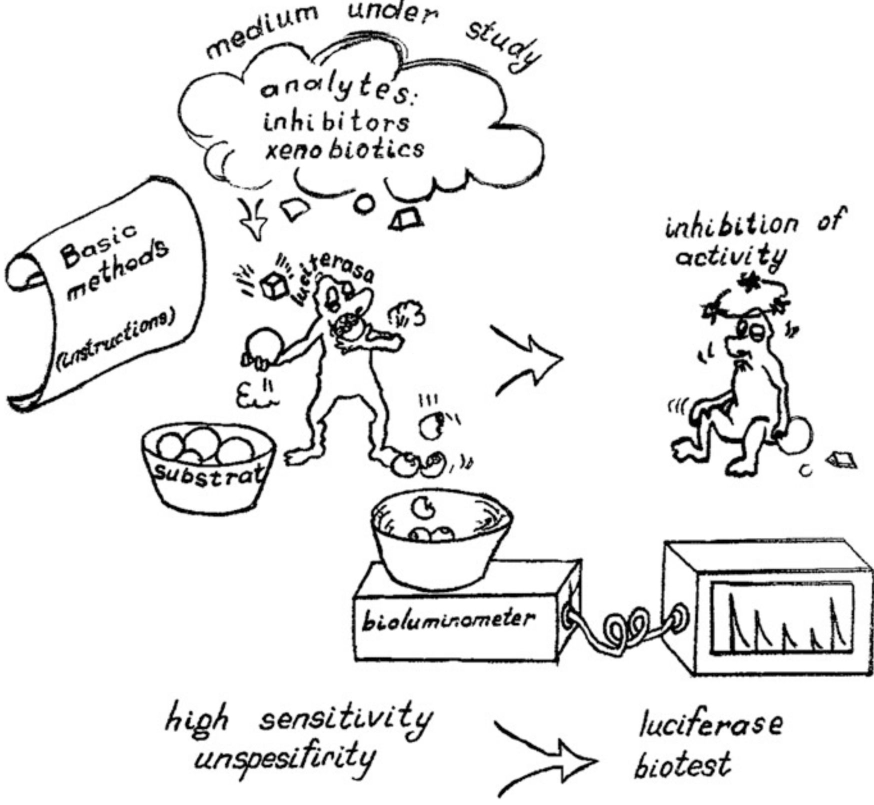
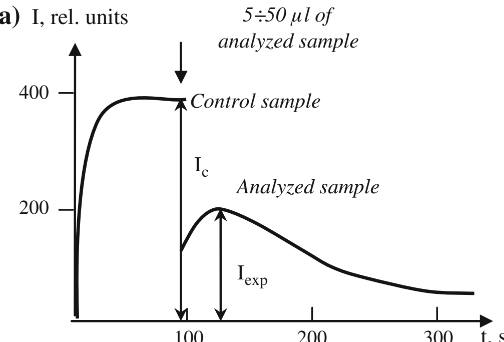


Fig. 1 Scheme of luciferase biotesting [11]

Enzymatic bioluminescent bioassays are based on the classification of bioluminescence inhibitors and activators according to the mechanism of their influence on fundamental physicochemical processes. The patterns of exogenous compound influence on bioluminescent systems are classified [19, 20]. According to this classification, there are four possible ways in which exogenous compounds act on a bioluminescent system: (1) influence of molecules on energy transport processes in a bioluminescent system, (2) influence of molecules on hydrogen transport processes in a bioluminescent system, (3) influence of molecules on electron transfer processes in a bioluminescent system, and (4) interaction of molecules with the enzymes of a bioluminescent system. Knowing the physicochemical basics of luminescent biotesting, it is possible to predict the analysis results and change the sensitivity of bioassays to certain pollutant groups [17, 18, 21–23].

When enzymatic biotesting is carried out, several analysis schemes can be used (Fig. 2). The first bioluminescent enzymatic bioassay variant is the quickest and demonstrates good repeatability of results. The following sequence of operations is performed: a cuvette with all the necessary components of a coupled enzyme



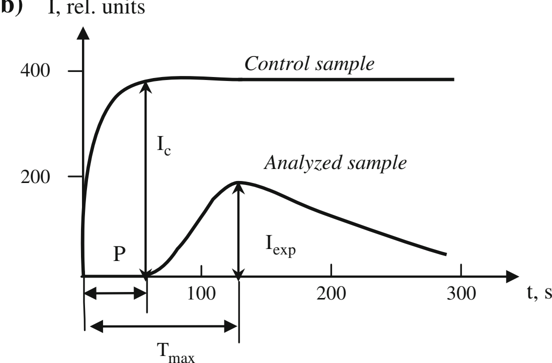


Fig. 2 Bioluminescent testing diagram (**a**); modified diagram of bioluminescent testing (**b**)

system of luminous bacteria, their substrates, and buffer solution are placed into a bioluminometer, and after the maximum light emission intensity I_c (control) is registered, from 5 to 50 μL of the test water sample or toxic substance solution are added, and the maximum light emission intensity I_{exp} is registered again (Fig. 2a).

The intensity of the residual luminescence $(I_{exp}/I_c) \cdot 100\ \%$ and the decay constant k_d are estimated. The decay constant is calculated according to the following formula: $k_d = [\ln(I_2/I_1)]/\Delta t$, where I_1 is the peak of bioluminescence intensity, I_2 is the bioluminescence intensity at the certain moment of time after reaching the bioluminescence maximum, and Δt is the time (minutes) needed for I_1 to reach I_2.

When analyzing toxicity of the water samples, the luciferase index (*LI*) or toxicity coefficient (*TC*) are calculated according to the formulas:

$$LI = \left(I_{exp}/I_c\right) \cdot 100\,\%,$$
$$TC = \left[\left(I_c - I_{exp}\right)/I_c\right] \cdot 100\,\%.$$

LI and *TC* are the residual luminescence and the degree of inhibition of the bacterial coupled enzyme system NADH:FMN-oxidoreductase-luciferase in the presence of analyzed sample correspondingly and $TC = 100 - LI$.

The criterion of toxicity is a 50 % decrease in the maximum luminescence level of the bacterial coupled enzyme system after the analyzed sample is added, as compared to the luminescence level in the control sample.

In the second case it is possible to achieve higher sensitivity of assays to the toxic substances. In this variant, testing of the control sample (usually distilled water or buffer solution) and analysis sample is performed in different cuvettes [24]. The reaction of the bioassay is also determined by the values of TC and k_d. But in that case, it is possible to use one more parameter for toxicity analysis: the time when the coupled enzyme system reached the luminescence maximum (T_{max}; Fig. 2b).

Application of the bacterial coupled enzyme system L + R for ecological monitoring is justified in quite a large number of works [18, 25, 26]. Bioluminescent bioassays based on the coupled enzyme system L + R were successfully used for the analysis of water in the Yenissei River and its tributaries, drinking water in various districts of Krasnoyarsk and Altai Territories, the salt lake Shira, as well as in Lake Baikal [27–29].

Bioassay reaction was studied in the analysis of potable and surface waters in the Altai region exposed to Semipalatinsk explosions and surface waters of the Yenissei River in the radioactive waste discharge area of the Mining-Chemical Combine (Krasnoyarsk) [26]. More than 100 samples of surface water and 170 water samples from wells and water supply systems have been analyzed. Results of the bioluminescent assay were compared with integral tests to assess surface water quality for its redox characteristics including hydrogen peroxide concentration, redox-status of aquatic medium, and OH-radical formation rate and lifetime. The assay makes possible to characterize water quality by three cleanliness classes according to PD 53.18.24.83-89 and define pollution (chemical or biological) causes [30]. In all parts of the Altai region the condition of surface and potable water caused concern. Results of the bioluminescent assay were confirmed by chemical and spectral analyses. In most samples results of bioluminescent assays have been found to correlate with the integral assay based on redox characteristics of the analyzed water [26].

Vetrova et al. [29] used bioluminescence bioassays based on luminous bacteria (*Photobacterium phosphopreum*) and adapted coupled enzyme system NADH:FMN-oxidoreductase-luciferase for monitoring the saline-water conditions of Lake Shira (Khakasia, Siberia). The saltwater Lake Shira has become a very popular health resort in southern Siberia, and the ecological problems of the lake are currently of great concern. The differences in bioluminescence responses have been found to be related to the salt composition and the oxidation–reduction properties of the water. Bioluminescent kinetics parameters, which are mostly sensitive to pollution under conditions of saline water, have been observed. The enzymatic system in the presence of 1,4-benzoquinone are shown to be more sensitive to redox characteristics of the salt water than in the absence of 1,4-benzoquinone. Thus, 1,4-benzoquinone should be included in a model solution for monitoring redox properties of the salt water. Using this technique, the results of bioluminescence analysis are used to construct a heterogeneity map that

characterizes the spatial and temporal water quality of Lake Shira. A partial map was based on the bioluminescence characteristics of water samples taken along the shoreline, sampling stations in the different places, and in different depths of the lake. The map reflects the correlation between the effect of the water samples on bioluminescence intensity and their chemical and physical–chemical characteristics. It has been demonstrated that the bioluminescence assay measurements must be done within 2 h after the sampling time.

The advantages of enzymatic assays based on the bacterial coupled enzyme system L + R are their rapidity (the time of analysis does not exceed 3–5 min), high sensitivity, simple measuring procedure, possibility of automation of ecological monitoring procedure, availability and safety of reagents, and a wide market of bioluminometers [31].

They were so easy to use and convenient that they started to be applied in the educational process, mainly in ecology practical courses [32–35].

2.2 Enzymatic Reagents for Bioluminescent Analysis

2.2.1 Ways to Obtain Stable Enzyme Preparations

Widespread use of the existing bioluminescent analytic methods is hampered by several disadvantages, including the instability of enzyme systems during use, limited shelf-life of enzymes–reagents for analysis, the need to control ambient conditions (i.e., pH, temperature, etc.) and interference by substances in the test samples, high manufacturing cost, and other factors [36].

These problems can be solved by using immobilized enzyme preparations in bioluminescent analysis, by obtaining reagents in the microenvironment that possess high catalytic activity and are stable for long-term storage, and making it possible to computerize analyses as biological modules of biosensors. Immobilization is the most effective and popular way among many known methods of enzyme stabilization. Advances in obtaining highly stable immobilized enzymes served as the basis for development of one of the foremost leading trends in biotechnology: creation of biosensors [37].

For the last 30 years immobilization has been widely used for production of stable reagents for bioluminescent analysis based on various bioluminescent systems: luminous bacteria, and bacterial and firefly luciferases. Many of the available immobilized reagents are successfully used in analytic measurements and in biosensors, because they simplify the analysis procedure, sometimes enabling full automation. At present, there are more than 40 different methods of immobilizing luminous organisms and enzymes isolated from various organisms [38].

The area of application of these methods is extremely wide: from analytical chemistry (analysis of NADH, FMN, ATP, and other substances), medicine (analysis of D- and L-lactate, bile acids in blood serum, amino acids such as alanine and phenylalanine, NADH-dependent enzymes, etc.), food industry (analysis of

the degree of bacterial content), ecological monitoring, and scientific research dealing with cell biology and in vivo enzymatic processes.

Production of immobilized reagents for analytic purposes is one of the major focal areas in applied enzymology. An important advantage of immobilized enzymes is the possibility to control the enzyme stability to physical and chemical environmental factors by way of choosing a suitable microenvironment. In an optimal microenvironment immobilized enzymes maintain high activity in a wide temperature range, and expansion of the range of pH optimum and ionic strength optimum is observed.

Table 1 shows the most effective methods of bioluminescent systems immobilization, and different immobilization methods are compared to reveal the advantages and disadvantages of each of them [38]. The activity of immobilized enzymes depends on the conditions under which the reagent was made. The optimal microenvironment for bacterial luciferase is natural polymer gel. To make a gel, gelatin or starch (potato, rice, or corn) can be used. By varying gel concentration, time, and mode of drying of immobilized enzymes it is possible to make reagents with different enzymatic activity [39–41].

The immobilized coupled enzyme system L + R does not require special storage conditions to save high enzymatic activity: when immobilized in starch or gelatin gel, it maintains its activity for 2 years [42]. Moreover, immobilization in starch and gelatin gel leads to a considerable stabilization of the coupled enzyme system with regard to denaturation treatment: pH optimum of the enzymes expands both to the acid and alkaline areas; high enzyme activity is maintained at increased salt concentration; thermal stability increases [43, 44]. The highest thermal stability is achieved in the coupled enzyme system immobilized in starch gel [40].

Apart from reagents including NADH:FMN-oxidoreductase and luciferase, there are reagents that include not only enzymes, but also the substrates of bacterial bioluminescent reaction. A patented stabilization and immobilization process preserves up to 50 % of the enzymatic activity and produces the homogeneous multicomponent reagent Enzymolum, which contains the bacterial luciferase, NADH:FMN-oxidoreductase and their substrates, coimmobilized in starch or gelatin gel [45]. The reagent is currently produced in tablet form and can be used in the cuvette variant of a bioluminometer (Fig. 3). The other forms (e.g. on the plane table, strips, and others) were also obtained for bioluminescent analysis. Enzymolum can be integrated as a biological module into the portable biodetector–biosensor of original construction.

Despite the significant advantages of immobilized reagents over soluble enzymes, the use of Enzymolum in toxicological studies was hardly possible at first, as the sensitivity of the bioluminescent coupled enzymatic system is not sufficient for testing pollutants at the maximum permissible concentration (MPC) level [17]. However, there are ways of increasing its sensitivity. This could be done by varying the conditions of testing, which is not possible for assays based on living organisms.

Table 1 Main features of different immobilization methods [38]

Polymer carrier	Relatively easy	Initial activity %[a]	Percentage activity at 4 °C after (days)[b]	Performance[c]	Turnover cycles	Automation
Polyacryl-hydrazide	Moderate 1–2	60	100 (15)	G—	20	Yes
BrCN-sepharose	Moderate 3–6	10–90	90 (5)	G 3–5	700	Yes
BrCN-agarose	Moderate 3–6	20–30	Stable (30)	VG 3–5	700	Yes
Epoxy-agarose	Moderate 3–6	0.1–1	70 (14)	P 0.5	–	No
Porous arylamino glass rods or beads	Moderate 6–10	1–2	Stable (15–180)	G—	700	Yes
Nylon tube reactor	Complex 6–8	50–70	100 (60)	G >1	900	Yes
Glass strips	Easy 1–2	92–96	100 (60)	G 1	100	Yes
Cellulose film	Easy 1–2	3–8	–	G 1	–	Yes
Lipid films	Complex 5–10	0.1–1	Stable (1–7)	P—	–	No
Bovine serum albumin gel	Easy <1	0,3–8	100 (30–60)	—1	100	No
Insoluble collagen	Easy 1–2	100	70 (14) 20 (240)[d]	G—	Several	Yes
Starch gel	Easy 10–12	100	100 (360)	VG 0.1	100	Yes
Alginate gel	Easy 2–10	30–60	100 (1)	G—	None	No
Polyamide membranes	Moderate 2–4	3–10	100 (180)	—1	None	No

[a] Relative activity compared with soluble enzyme
[b] Percentage of initial activity after number of days at 4 °C
[c] Reproducibility: *G* good; *VG* very good; *P* poor, or sensitivity relative to soluble enzyme set 1
[d] High mechanical strength

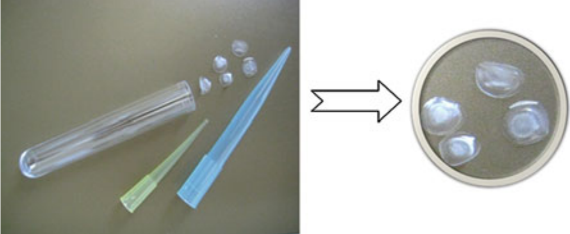


Fig. 3 The multicomponent reagent Enzymolum is a disk of dried film, diameter 6–7 mm; dry weight 1.5 ± 0.2 mg

2.2.2 Design of the Reagent Enzymolum and the Procedure of Toxicological Bioassay with Increased Sensitivity to Pollutants

The following methods were tested to increase the sensitivity of the reagent to pollutants: varying the composition of the immobilized reagent; varying the ratio of reaction mixture and test sample; varying the order of components added to the coupled enzymatic system and test sample; introducing an additional step of reagent incubation to the test water sample; and selecting the control solution for biotesting [46].

Varying the Composition of the Immobilized Reagent

Information about the dependence of bioluminescence kinetic parameters on the reaction mixture ratio, the concentration of enzymes and substrates, and enzyme stabilizers made it possible to increase the sensitivity of the bioassays. We studied the dependence of the sensitivity of NADH:FMN-oxidoreductase and luciferase immobilized in starch gel with the substrates of NADH and tetradecanal to the effect of benzoquinone and $CuSO_4$ upon the concentration of the immobilized reagent components. Figure 4 shows that the sensitivity of the multicomponent reagent increased as the enzyme concentration of its content decreased. A reagent with luciferase content 0.2 μg gave the greatest sensitivity: the residual luminescence intensity was 20–30 %. Changes in the other bioluminescence parameters (T_{max} and k_d), caused by varying the enzyme content in the multicomponent reagent, were insignificant. Increasing the NADH content in the reagent also decreased the multicomponent reagent's sensitivity to the effect of the analyzed pollutants. The maximum sensitivity to the effect of the analyzed substances was observed when the NADH content in the reagent was 0.1 mM (Fig. 4).

To prevent inactivation enzymes and to maintain their activity during use and storage, enzyme stabilizers with various mechanisms, including stabilizers of enzyme's SH-groups (DTT or mercaptoethanol) and BSA, were introduced to the multicomponent reagent. Introducing enzyme-stabilizing additives to the immobilized reagent increased the activity of the coupled enzymatic system L + R and the stability of immobilized enzymes during storage, but decreased the reagent's sensitivity to toxic substances (Fig. 5).

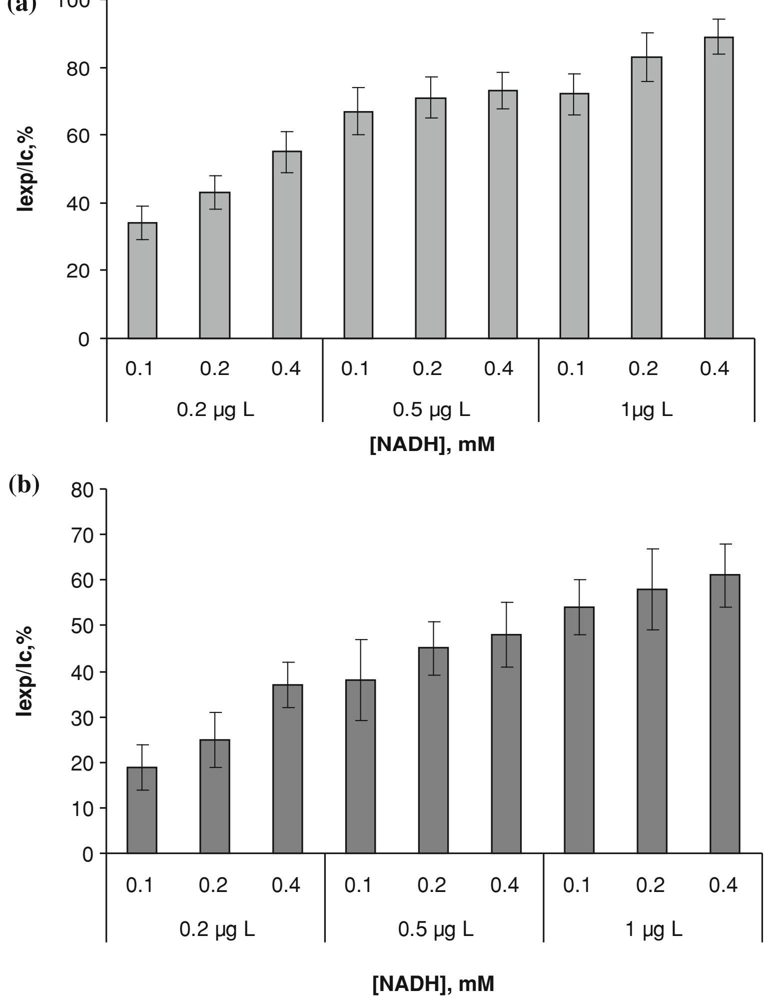


Fig. 4 Residual light intensity of immobilized reagent under variation of luciferase (*L*) and *NADH* content in reagent in the presence of 50 μg L^{-1} benzoquinone (**a**) or 5 μg L^{-1} $CuSO_4$ (**b**) [46]

It was shown previously [39] that introducing FMN into the immobilized reagent resulted in a significant decrease in the activity of the coupled enzymatic system; that is why FMN was not included in the reagent and FMN solution of various concentrations was added during the analysis. When the FMN concentration in the reaction mixture was increased, the sensitivity of the multicomponent reagent to benzoquinone decreased and remained at the same level to $CuSO_4$ (Fig. 6).

Thus, it is shown that by reducing the content of any component in the immobilized reagent, it is possible to increase the reagent's sensitivity to toxic

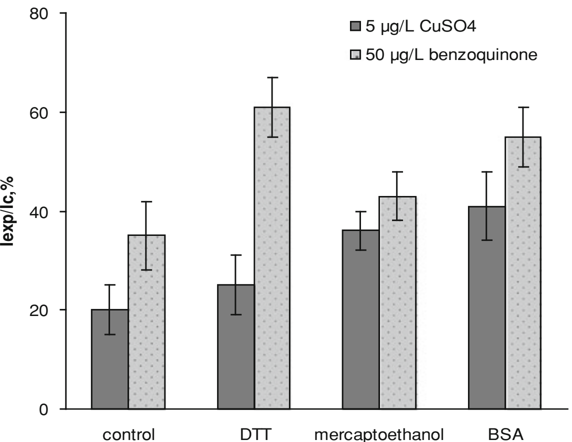


Fig. 5 Residual light intensity of immobilized reagent under variation of its content in the presence of 5 μg L^{-1} $CuSO_4$ or 50 μg L^{-1} benzoquinone. Content of stabilizers in disks was: *DTT* 0.1 mM, *mercaptoethanol* 0.2 mM, *BSA* 0.001 mM. *Control* is the reagent without any stabilizer [46]

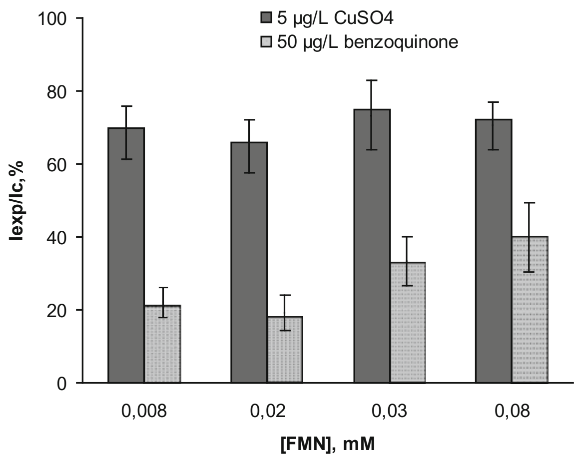


Fig. 6 Residual light intensity of immobilized reagent under variation of FMN concentration in the presence of 5 μg L^{-1} *$CuSO_4$* or 50 μg L^{-1} *benzoquinone*. *FMN* was injected as a solution [46]

substances. This effect can be explained by the fact that the coupled enzyme system had nonstationary conditions because the reagent contains a nonsaturating concentration of one of the substrates (NADH). Indeed, the maximum sensitivity of the coupled enzyme system to benzoquinone and $CuSO_4$ was observed when the NADH concentration was 1.3 times lower than the one corresponding to the Michaelis constant for this substrate [40].

Varying the Order of Components Added to the Reaction Mixture

In all the experiments described above, the pollutant solution was added to the reaction mixture after the maximum luminescence level had been reached, that is, after the substrates and enzyme active centers had interacted and, probably,

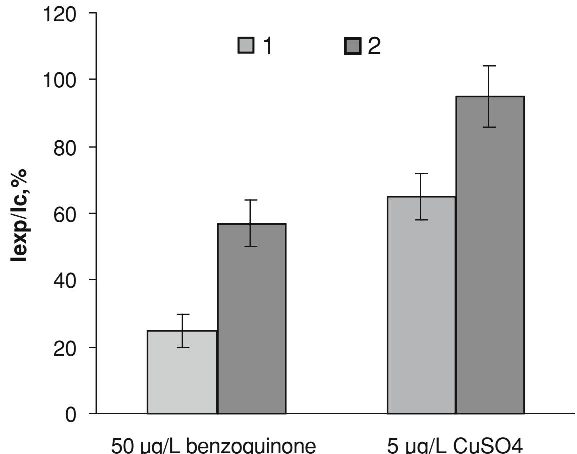


Fig. 7 Residual light intensity of immobilized reagent under variation of the order of introduction of the bioassay's components into the reaction mixture in the presence of $CuSO_4$ or benzoquinone: *1* introduction of analyzed water sample before starting enzyme reactions by FMN; *2* introduction of analyzed water sample after light intensity reaches its maximum [46]

formed enzyme–substrate complexes. The sensitivity of the coupled enzyme system can apparently be increased by injecting the analyzed sample before the maximum luminescence level has been reached, that is, before adding the last reaction substrate. In this case the increase in the reagent's sensitivity is explained by the enhancing of competition between the substrates and the added inhibitor toxicants.

Figure 7 shows that adding the analyzed sample before starting the reactions by the FMN solution led to an insignificant increase in the sensitivity of the immobilized coupled enzyme system to benzoquinone and $CuSO_4$.

Incubating Enzymes in the Test Solutions

The multicomponent reagent with the control or analyzed pollutant solution was incubated for 30–900 s, then the reaction was started with the FMN solution, and I_c and I_{exp} were measured (Fig. 8).

It was shown that such additional incubation increases the sensitivity of the immobilized reagent to copper sulfate, and the sensitivity to benzoquinone remains at the same level. The inhibition of enzymes with the copper sulfate solution grew proportionally to the increase of incubation time and reached its peak when the reagent had been incubated for 120–130 s with little effect on the assay sensitivity at 300-s incubation. A further increase of incubation time is impossible because the activity of the immobilized reagent is significantly lowered, even when the control solution is analyzed.

Thus, we found the conditions for biotesting aqueous solutions using the immobilized reagent that provide the maximum sensitivity to toxic substances.

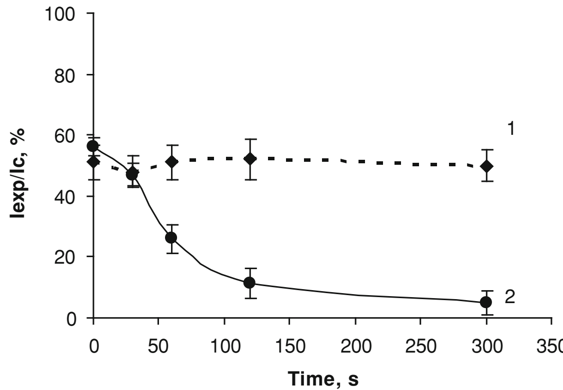


Fig. 8 Residual light intensity of immobilized reagent under variation of the time of reagent incubation in benzoquinone (*1*) or $CuSO_4$ (*2*) solutions [46]

Table 2 Effects of some pollutants on bioluminescence of soluble and immobilized coupled enzyme system NADH:FMN-oxidoreductase and luciferase [46]

Class	Substance	MPC, mg L^{-1}	EC_{50}, mg L^{-1}	
			Soluble L + R	Coimmobilized L + R, C14, NADH
Quinones	Benzoquinone	0.1	0.05	0.01
	Naphtoquinone	0.25	0.003	0.003
	Thymoquinone	*	0.005	0.03
	Toluquinone	*	0.01	0.05
Phenols	Hydroquinone	0.2	0.03	0.02
	Pyrocatechin	0.1	2.2	0.002
Heavy metal salts	$CuSO_4$	1	0.2	0.002
	$CdSO_4$	0.001	310	0.08
	CoC_{12}	0.1	40	0.1

*MPC isn't determined

Comparing Sensitivity of Soluble and Multicomponent Immobilized Coupled Enzyme System NADH:FMN-Oxidoreductase-Luciferase

The effect of pollutants upon the bioluminescent system was estimated according to the parameter EC_{50} which is the concentration of the active substance when bioluminescence is inhibited by 50 %. This parameter was chosen by analogy with the generally accepted parameter EC_{50} which is the effective concentration of the active substance causing a 50 % change in some vital functions of the test organism [47]. The values of EC_{50} were determined for various classes of organic pollutants using the soluble and immobilized coupled enzyme system L + R (Table 2).

Table 2 shows that the sensitivity of the soluble coupled enzyme system L + R to the studied pollutants was higher as compared to that of the immobilized

reagent. However, because the immobilized reagent enables measurements at the MPC level or even lower for almost all the studied substances, it can be used for biotesting natural and waste waters.

2.3 *Biotesting Natural and Waste Waters Using the Soluble and Immobilized Coupled Enzyme System NADH: FMN-Oxidoreductase-Luciferase*

The enzyme biotesting approach was used as a platform technology to certify "Method to measure the intensity of bioluminescence with the help of the 'Enzymolum' reagent to detect the toxicity of drinking, natural, waste and treated waste water" [24, 48]. The biotesting method of using an immobilized reagent was tested in analyzing the wastewaters of the pulp and paper plant. The characteristics of the samples are given in Table 3, where Sample #1 is the wastewater coming to the department, Sample #2 is the wastewater after mechanical treatment, and Sample #3 is the purified water from the outlet at the treatment facilities.

The criterion of toxicity was a 50 % decrease in the maximum luminescence level of the reagent after the analyzed water sample was added, as compared to the luminescence level in the control sample. The water's quality was determined by its toxicological characteristics, using the value of the biologically safe dilution, established by Russian Federation standards, according to Table 4.

The biotesting values were analyzed after samples had been diluted with distilled water or phosphate buffer. It is common practice in biotesting to use distilled water for diluting water samples, which makes it difficult to work with soluble enzymes. For example, there is a high level of luminescence inhibition by the control solution, distilled water, because of the discrepancy between the pH of distilled water (about 5.5) and the optimum pH of the coupled enzymatic system (about 7.0). Using 0.05 M potassium-phosphate buffer with pH 7.0 as the control solution can lead to a misinterpretation of biotesting results. If the environment studied is polluted with heavy metals, phosphates contained in the buffer cause them to precipitate, which reduces the effect of heavy metals on the luminescence of the coupled enzymatic system L + R. However, when soluble enzymes were replaced with an immobilized reagent that had a wider pH optimum range (from 5.0 to 9.0) than had the enzyme solutions [40], no such effect was observed.

Table 5 presents the results of wastewater biotesting using the immobilized reagent and under the Mann–Whitney U test, showing no difference between a sample diluted by water or buffer. According to the results, samples #1 and #2 were acknowledged to be hypertoxic, and sample #3 to be toxic. In addition, it was shown that the samples' toxicity decreases in a row: Sample #1 > Sample #2 > Sample #3, which corresponds to the changes in the chemical characteristics of the analyzed samples. This experiment confirmed the appropriateness and

Table 3 Results of the chemical analyses of waste and purified water samples

Component	Sample #1	Sample #2	Sample #3
pH	6.5	6.2	3.3
COD[a], mg L^{-1}	3,000	2,200	2,000
Dredge, mg L^{-1}	300	74	51
NH^+, mg L^{-1}	66	53	48
Nitrites, mg L^{-1}	<0.02	<0.02	<0.02
Nitrates, mg L^{-1}	6.2	0.1	0.1

Waste and purified water samples from the laboratory of the industrial waste purification department at the pulp and paper plant where sample #1 is the wastewater, sample #2 is the wastewater after mechanical treatment and sample #3 is the purified water [46]

[a] Chemical oxygen demand

Table 4 Toxicological characteristics of analyzed water in accordance with its dilution factor [46]

Dilution factor of analyzed water	Toxicological characteristics of analyzed water
1	Nontoxic
2	Slightly toxic
From 3 to 10	Toxic
From 11 to 50	Highly toxic

Table 5 Results of the toxicity bioassay of waste water at pulp and paper plant

Test water sample	Toxicity coefficient, %	
	Buffer[a]	dH_2O[b]
Sample #1	100	100
Sample #1, diluted 10 times	90.9 ± 7.9	90.5 ± 8.2
Sample #1, diluted 100 times	34.1 ± 6.6	31.0 ± 7.2
Sample #2	100	100
Sample #2, diluted 10 times	80.3 ± 8.0	79.4 ± 7.9
Sample #2, diluted 100 times	58.6 ± 7.4	56.6 ± 6.5
Sample #3	±10.2	86.5 ± 8.9
Sample #3, diluted 10 times	22.4 ± 4.8	18.7 ± 5.1

[a] 0.05 M potassium-phosphate buffer with pH 7.0 was used as a control sample and to prepare the analyzed water sample dilutions

[b] Distilled water was used as a control sample and to prepare the analyzed water sample dilutions

efficiency of using the immobilized reagent instead of the soluble coupled enzyme system L + R in biotests.

Another example is concerned with water bodies containing clean "reference" water, such as Lake Baikal. During the studies of water from Lake Baikal a comparison was made of assays based on a soluble and immobilized coupled enzyme system depending on the place and depth of sampling (Fig. 9). Samples from five control points were studied.

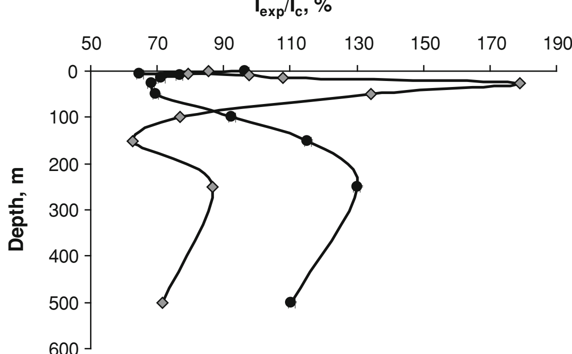


Fig. 9 Dependencies of the light emission intensity of a soluble (lyophilized) and immobilized couple enzyme system L + R on the depth of water sampling at Station 1 of Baikal lake

The nature of luminous intensity dependence for water samples from different depths was similar with both soluble and immobilized coupled enzyme systems. Water samples taken from the upper (the warmest) epilimnion zone didn't cause intense luminescence; it must be mentioned that, depending on the location of the sampling station, the zone was within the range of 5–50 m from the surface. Apparently, the slight stimulation effect could be explained by the fact that the epilimnion water, as compared to the other water body layers, contains more oxygen due to a larger phytoplankton biomass. Water samples from the depth of 50–100 m (depending on the place of sampling) had a slight effect on the luminous intensity with both soluble and immobilized coupled enzyme systems, which didn't suggest pollution, as the test readings were within the range of possible fluctuations of enzymatic assay parameters for "normal" unpolluted water.

2.4 The Reagent "Enzymolum" in Toxicological Bioassay of Air

Bioluminescent assays were used not only for aquatic environments, but also for monitoring of air pollution [49]. The sensitivity of luminous bacteria and their enzymes to air samples differing by industrial pollution degree was determined. Air samples were collected in the clean (Akademgorodok, sample #1) and polluted (the coal power plant area, sample #2) districts of the city of Krasnoyarsk. The air samples were collected into liquid absorption medium (water, ethanol, or acetone). The standard aspirating device performing 1.0 L/min was used. Chemical composition of the samples was analyzed with a gaseous chromatograph (Agilent Tech. 7890A). To compare the sensitivity of assays the numbers of dilution of the samples necessary to remove the toxic effect were considered (Table 6).

The results indicate that water is better than ethanol or acetone medium for air sample preparation because of its sufficient capacity to absorb organic compounds, and absence of interfering effects on bioluminescence. The sensitivity of soluble

Table 6 Comparative characteristics of bioluminescent assays used to monitor air pollution

Type of bioassay		Soluble coupled system	Luminous bacteria	Immobilized coupled system (Enzymolum)
Number of components (simplicity)		5	2	3
Duration of assay, min		10	5–30	7
Sensitivity (number of dilutions), sample#1/sample#2	Water	3/2000	3/700	3/16,000
	Ethanol	1/700	1/1	3/250
	Acetone	3/2,000	1/1	3/>2,000
Storage conditions		2 months at +15 °C, 3 years at –18 °C	6 months at +5 °C, 1 year at –18 °C	2 years at +4 to +25 °C

and immobilized enzymes is 3–24 times higher than the sensitivity of bacterial-based tests. The immobilized reagent provides the reduction of the time required to complete the analysis (down to 7 min), easy-to-use, higher sensitivity (allowed dilutions are up to 16,000), and the possibility to increase the volume of the sample up to 97 % of the total. Thus there is the possibility to apply the bioluminescent bioassays based on the immobilized reagent Enzymolum for air pollution monitoring [49].

2.5 Signal System of Enzymatic Assays: Methodological Aspects

In all the existing bioassays based on living organisms, the reaction of the test substance with the substances that are harmful for these objects is determined. In this context the term “environmental toxicity” is used, although it is not always correct. It is also considered that all living organisms, including humans, react with the test medium in a similar way. Such a conception is based on the statement that all living organisms have a similar structure and functions that are affected by toxic substances. The idea that normal vital activity of all living organisms—from amoebas to humans—is based on complex interconnected activity of many enzymatic systems providing with such functions as respiration, digestion, reproduction, bioluminescence, and the like, suggests that a universal test system could be developed consisting of a number of enzymatic reactions. Indeed, it is generally accepted that all living beings, despite the diversity of substrates involved in metabolism, including specific ones for a given organism, have similar basic types of reactions, and based on the activity of enzymes released under the effect of pollutants it is possible to determine the biochemical mechanisms of these substances affecting not only functions, but organisms on the whole.

Such a model enzymatic test system must include enzymes of different classes, or key enzymes of metabolic processes in living organisms. In this case it will be possible to determine which vital function of organisms will be inhibited by which toxic substance or their mixture.

Developing such a complex model test system is possible on the basis of bioluminescence. Using the coupling principle in bioluminescent analysis gives almost unlimited possibilities for determining the activity of various enzymes [5] and designing new enzymatic assays on this basis. The possible basis for development of new bioluminescent enzymatic bioassays is the coupled enzyme reaction NADH:FMN-oxidoreductase-luciferase.

The unique feature of the enzymatic analytical systems is that, due to the coupling with bacterial luciferase, it is possible to measure the activity of more than 100 enzymes [5] if enzyme coupling chains are created where the product of one enzyme is the substrate of the next one. The luciferase of luminous bacteria must be the terminal enzyme in coupling chains for such enzymes as lactic dehydrogenase, trypsin, glucose-6-phosphate dehydrogenase, and others, making it possible to measure the activity of many enzymes according to the luminous intensity.

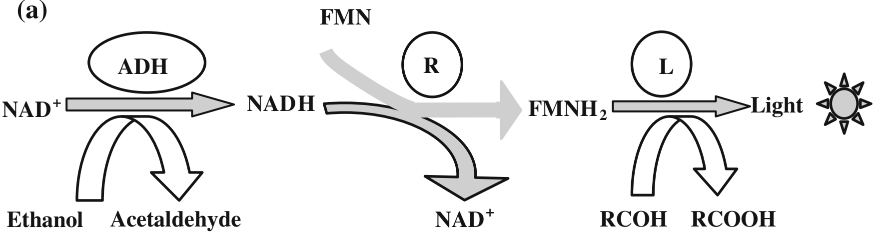
Moreover, using enzymatic reactions instead of living organisms in toxicity bioassays makes it possible to regulate the sensitivity of enzymatic assays depending on the objective. For example, the sensitivity of enzymatic assays to the toxic substances may be increased by extending the coupling chain of enzymatic reactions [50].

As a result of using several chains of coupling enzymatic reactions with bacterial bioluminescence, a set of enzymatic assays sensitive to different groups of pollutants was created as a “signal system of enzymatic assays” [25, 51].

2.6 How to Design New Bioluminescent Enzymatic Bioassays

To develop new bioluminescent enzymatic assays, the following methods were suggested and used: extending the chain of enzyme coupling and using different enzyme interaction mechanisms and types. As an example, two enzymes were chosen: alcohol dehydrogenase (ADH) and trypsin [25]. These objects were chosen, firstly, because they belong to different classes (oxidoreductases and hydrolases), and secondly, because they interact differently with bacterial luciferase, providing different sensitivity to the effect of toxic substances [50, 52].

Next we are going to look at the principles of designing enzymatic bioluminescent assays in more detail. The easiest way to create an enzymatic bioassay is to use direct coupling of enzymatic reactions. Such a method of assay construction by making long enzyme coupling chains has been known for quite a long time. At first a bioassay was developed based on the monoenzymatic reaction of luminous



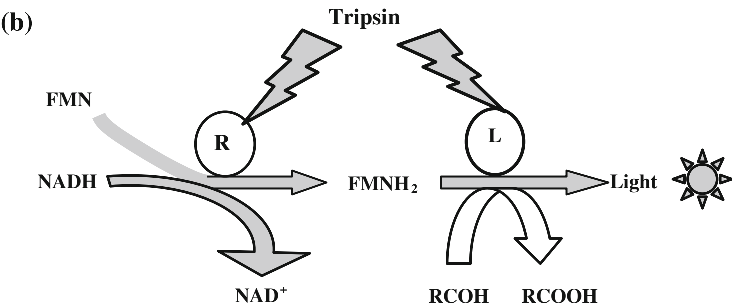


Fig. 10 Examples of coupling of the enzymatic reactions

bacteria (Reaction 1). Later a coupled enzymatic bioassay was created by coupling reaction (1) with NADH:FMN-oxidoreductase (Reaction 2) [11]. In the work of Kudryasheva et al. [50], three enzymes were coupled with direct ADH reaction as an example. In this case, NAD^+ was added to the reaction mixture that was subject to enzymatic reduction (Reaction 3) catalyzed by ADH, the resulting product NADH entered Reaction (2), and then other stages of the light emission process occurred.

$$\underset{}{\overset{ADH}{C_2H_5OH + NAD^+ \Leftrightarrow NADH + H^+ + C_2H_4O}} \tag{3}$$

The luminous intensity during Reaction (1) is proportional to the concentration of $FMNH_2$ resulting from Reaction (2); the concentration of NADH depends on the activity of ADH in Reaction (3; Fig. 10a). It should be noted that bioluminescent methods used in enzyme activity measurement make it possible to couple more than three enzymes, thus enabling development of new enzymatic assays that can be applied for the analysis of toxicity of different samples.

Apart from direct coupling, there are other types of interaction between enzymes that can be used for the development of enzymatic bioassays. Using the example of reverse ADH-reaction (Reaction 3), a method was suggested for the creation of a triple enzymatic bioassay by adding a NADH-dependent reaction to the coupled enzyme system.

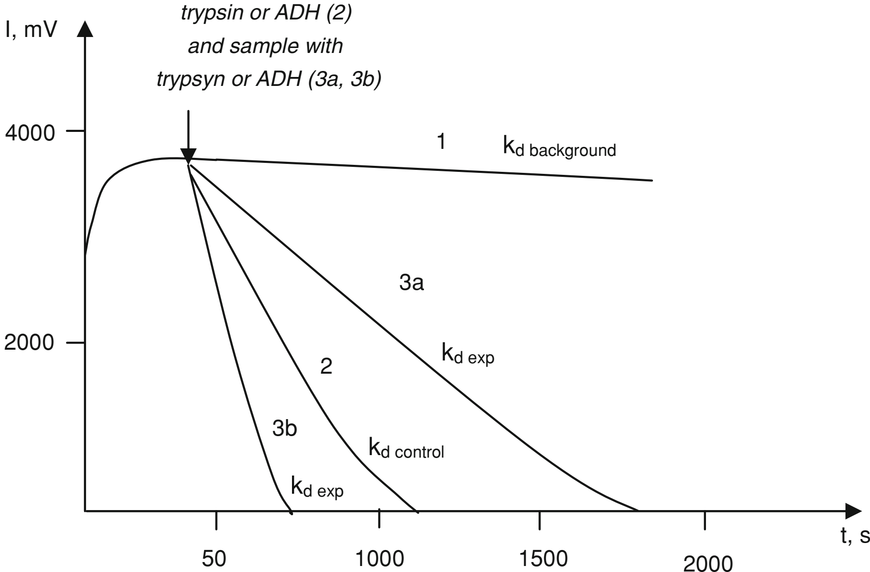


Fig. 11 Scheme of ADH (or trypsin) activity measurement using the bioluminescence decay constant: $k_{d\ background}$ is the decay constant for the coupled enzyme Reaction (*1*); $k_{d\ control}$ is the decay constant for the triple enzyme reaction with ADH or trypsin in the absence of pollutant (*2*); k_{dexp} is the decay constant for the triple enzyme reaction with ADH or trypsin in the presence of pollutant. *3a* the pollutant inhibits trypsin or ADH activity; *3b* the pollutant activates trypsin or ADH activity

The method determined the activity of NADH-dependent dehydrogenases, based on the measurement of the rate of reverse reaction catalyzed by dehydrogenase, which was developed by Petushkov et al. [53]. When a constant level of light is reached, acetic aldehyde and ADH are added to the coupled enzyme reaction L + R. Then, based on the quasi-first-order constant of bioluminescence recession proportional to the concentration of NADH, the activity of ADH is calculated (Fig. 11). This method is 20 times more sensitive than spectrometric measurements. The method is simple, one-stage, and the analysis time is 2–3 min. Similar methods have been developed to determine the activity of a number of other NADH-dependent dehydrogenases. All of them can be used as enzymatic bioassays.

The third way of designing enzymatic bioassays is based on the bioluminescent method used to measure the activity of proteolytic enzymes (Fig. 10b). It is based on the method of measuring the activity of proteases according to the bioluminescence recession constant [54]. When proteolytic enzymes are added to the reaction mixture, hydrolysis of luciferase and NADH:FMN-oxidoreductase occurs, causing a sharp decrease in luminous intensity (Fig. 11). Kudryasheva et al. [52] studied the effect of anthropogenic pollutants on proteolytic enzymes using trypsin as an example.

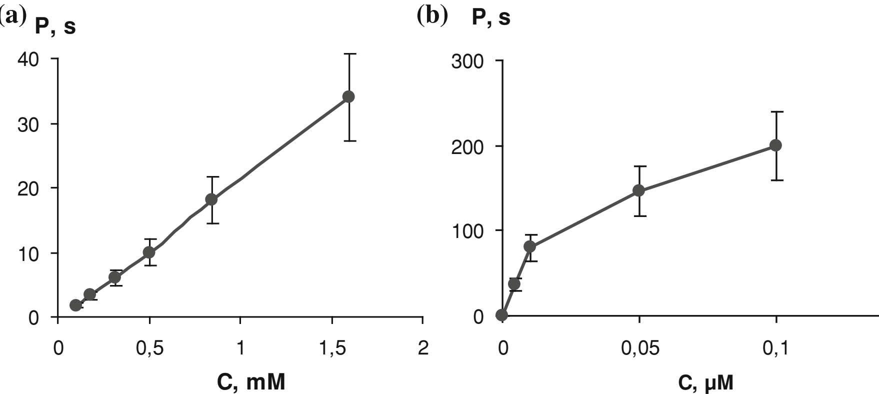


Fig. 12 Dependence of induction period on the concentration of 1,4-benzoquinone in the reaction mixture, **a** for the coupled enzyme system L + R, and **b** for the triple enzyme system ADH + L + R. Increasing the number of links in the coupling chain of enzymatic reactions results in a tenfold increase of sensitivity to benzoquinone [50]

Thus, estimation of the effect of chemical substances on the activity of the triple enzyme system with ADH and trypsin is based on changes in kinetic parameters of bioluminescence (Fig. 11). The procedure for triple enzyme biotesting is as follows.

- The maximum luminescence intensity of the coupled enzyme system L + R is measured, and then its luminescence intensity decrease is measured for 1 min. The decay constant ($k_{d\ background}$) for the case of the coupled enzyme reaction is calculated by the formula: $k_{d\ background} = \ln(I_{max}/I_1)/(t_1 - t_{max})$, where I_{max} is the bioluminescence intensity maximum and I_1 is the bioluminescence intensity at the moment of time t_1 (Fig. 11).
- 5–50-μL test sample and 5-μL ADH or trypsin solution are added to the reaction mixture described above. Then the decay constant $k_{d\ exp}$ is measured for the triple enzyme reaction.
- The authentic decay constant for the triple enzyme reaction is calculated by the equation: $k_d = k_{d\ exp} - k_{d\ background}$.
- For control measurement, 5-μL ADH or trypsin solution and 5–50-μL phosphate buffer (0.05 mol L^{-1}, pH 6.8) or distilled water are added to the reaction mixture described above. The control decay constant, $k_{d\ control}$, is calculated.
- Relative activity of ADH or trypsin is calculated using the equation: $A = (k_d/k_{d\ control}) \times 100\ \%$. The obtained value is proportional to the sample's toxicity.

Thus, three types of enzyme interaction were suggested for development of a set of enzymatic bioluminescent bioassays: direct reaction coupling, competition-for-substrate method, and proteolytic interactions. It was demonstrated that using different types of interactions it is possible to create enzymatic assays differing by their sensitivity to toxic substances. Kudryasheva et al. [50] showed that increasing

the number of links in the coupling chain of enzymatic reactions results in a tenfold increase of sensitivity to benzoquinone (Fig. 12).

The signal system of enzymatic bioassays was optimized and used for monitoring of natural and laboratory aquatic ecosystems and for studying the seasonal dynamics of zooplankton nonconsumptive mortality [55], as well as for toxicity analysis of pesticides [51] and sanitary assessment of natural polymers polyhydroxyalkanoates [56] and other applications.

2.7 Signal System of Enzymatic Methods for Toxicological Bioassay of Natural and Laboratory Aquatic Ecosystems

A signal system of bioluminescent assays was developed to monitor water quality in natural and laboratory ecosystems. It consisted of three enzymatic systems: coupled enzyme system NADH:FMN-oxidoreductase-luciferase and triple enzyme systems with alcohol dehydrogenase and trypsin. The results were compared with those for luminous bacteria.

The set of bioassays was applied to a small forest pond (Siberia, Russia), laboratory microecosystems polluted with benzoquinone and a batch culture of blue-green algae [25].

2.7.1 Dynamics of the Pond Ecosystem and the Microecosystem Components and the Bioassay Data

Prior to using these bioluminescent bioassays for ecological monitoring of water, it is necessary to answer the question: are the assays too sensitive; that is, do they respond to a natural variability of ecosystems, which is not connected to any pollution? That is why first the response of each bioassay to the water of the pond not subjected to heavy pollution was determined. The responses of the four bioassays for two extremely different parts of the ecosystem, epilimnion and hypolimnion, were compared [25].

The study was carried out at a small freshwater pond situated in a forest in the vicinity of Akademgorodok (Academic Town) of the city of Krasnoyarsk (Siberia, Russia). The pond has an oval shape, its surface area is about 3,000 m^2 and its maximum depth is 9 m. The samples were collected from epilimnion (depth about 2 m) and hypolimnion (depth about 7 ± 8 m) twice a week during three summer months.

Water temperature in the pond had ordinary summer variations (Fig. 13). A pronounced thermocline occurred at the depth of about 4 m in June–July and the temperature of hypolimnion (the layer below the thermocline) was 4 ± 10 °C lower than that of the epilimnion (the layer above the thermocline). In August the decrease of depth resulted in diminishing the difference between the epilimnion

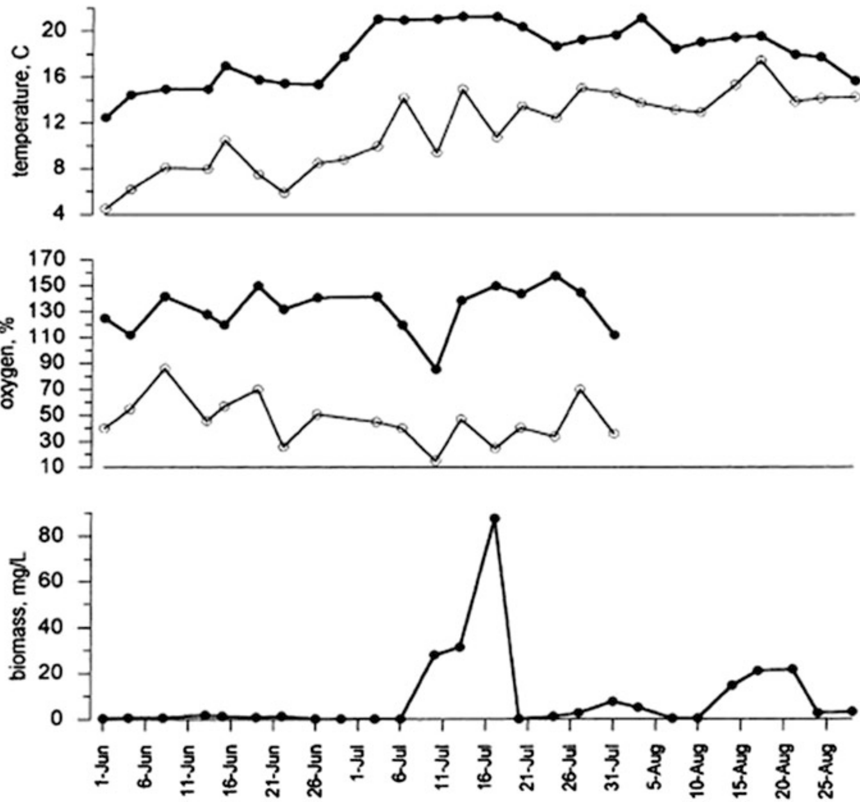


Fig. 13 Dynamics of the pond ecosystem components: (●) epilimnion, (○) hypolimnion [25]

and hypolimnion temperature. Dissolved oxygen saturation values (Fig. 13) in the epilimnion of the pond were significantly higher than those in the hypolimnion. In phytoplankton, green algae *Volvox aureus* Ehr. and diatom *Cyclotella comta* (Ehr.) Kutz. dominated. Blooms of *V. aureus* took place in the middle of July and in the middle of August (Fig. 13). *C. comta* dominated in the middle of June, when the water temperature was comparatively low. At the end of July and beginning of August, a small decrease of water temperature took place (Fig. 13) and *C. comta* became dominant in this period. In the hypolimnion only two samples of phytoplankton were taken in June and July, but the other dominant diatom and green species then in the epilimnion, *Stephanodiscus hantzschii* Grun., and *Closterium peracerozum* were found in the samples. Thus, the epilimnion and hypolimnion represented separate compartments of the pond ecosystems and naturally differed in water quality.

The variations of enzyme bioassay data were insignificant during the study period (Fig. 14). The data for the luminous bacteria based assay varied over the season. Water samples taken from the hypolimnion had a stronger inhibitory effect on bacterial luminescence.

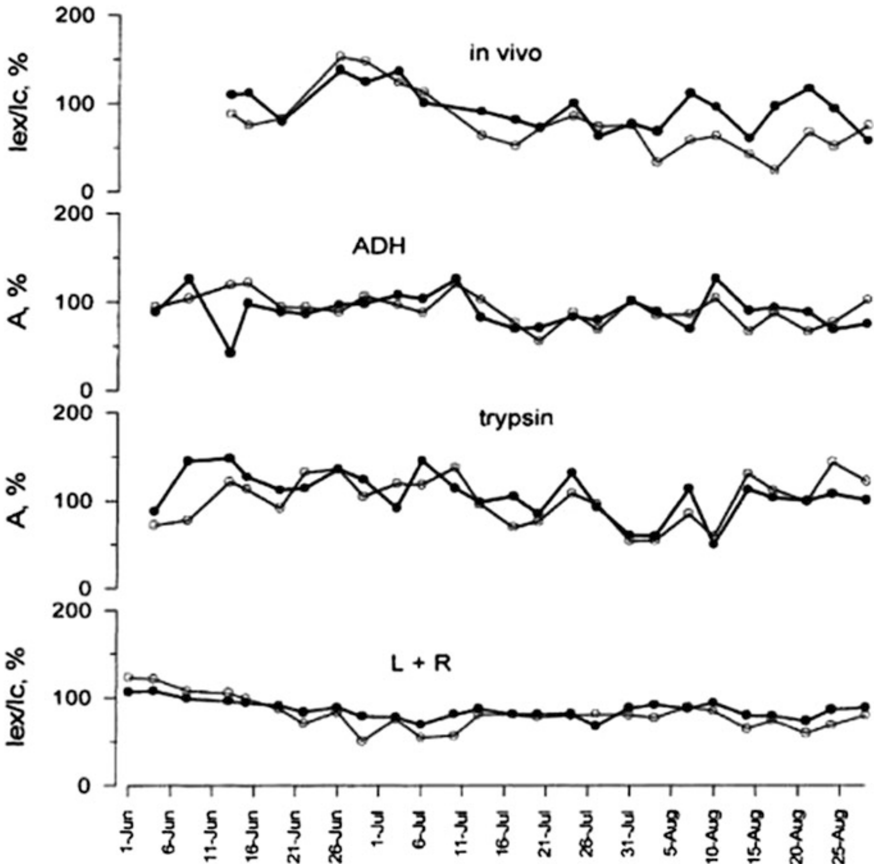


Fig. 14 Parameters of bioluminescent bioassays for the study of water from local pond: (●) epilimnion, (○) hypolimnion. A, %: relative activity of ADH and trypsin, I_c and I_{ex}: light intensity in control and experimental cuvette, respectively, for NADH:FMN-oxidoreductase-luciferase and luminous bacteria bioassays [25]

Only the bioassay based on the luminous bacteria demonstrated the significant difference between the epilimnion and hypolimnion by the Wilcoxon test. For the assays based on enzyme systems, the differences between the epilimnion and hypolimnion were insignificant.

At the end of summer water samples from the pond epilimnion (depth of about 2 ± 3 m) were inoculated into three cylindrical glass experimental microecosystems (MES) 25 cm in diameter and 25 cm high. They were illuminated by white light of 2.5 W/m^2 (natural intensity at the depth of sampling). Water samples from MES were taken twice a week for 2 months. Benzoquinone was added in the last week of study to MES 1.

The MES's experiment consists of two parts. In the first part (lasting 42 days; Fig. 15a) the relatively higher biomass of microalgae from the pond was

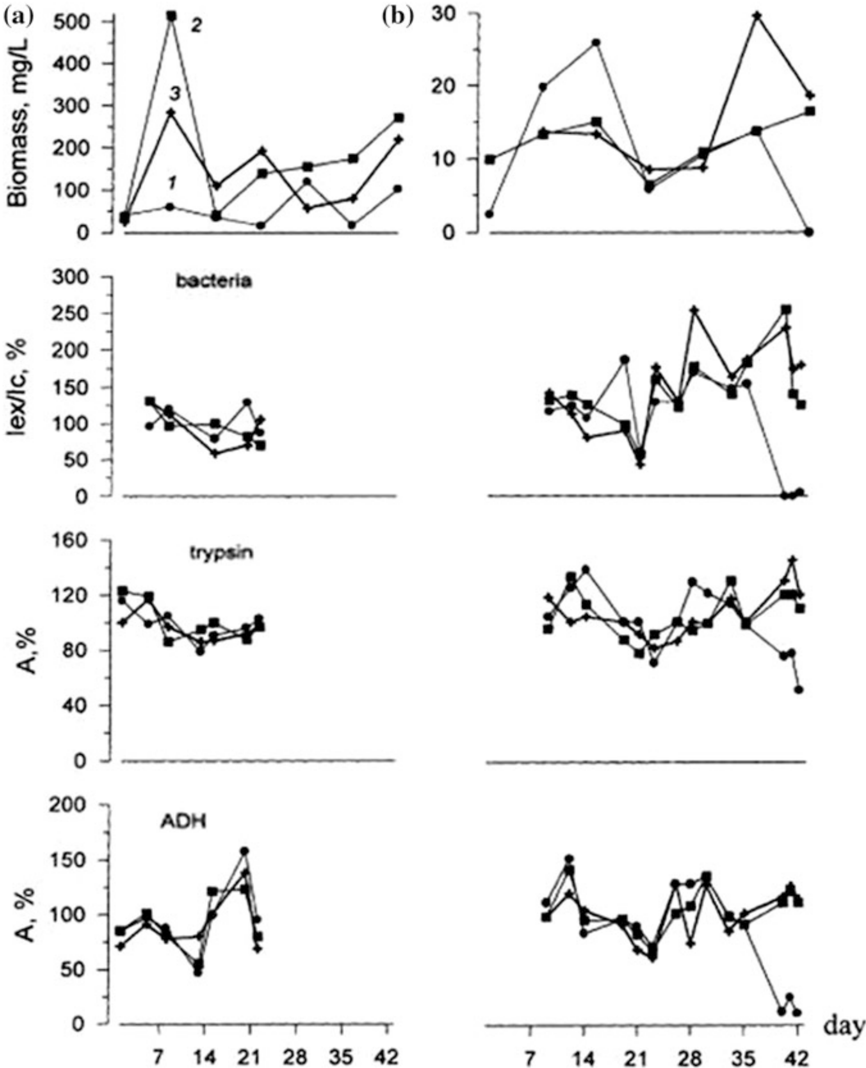


Fig. 15 Biomass of algae and the dynamics of bioassay parameters for MES: (●) MES 1 (treatment with benzoquinone on the 35th day of experiment (**b**), (■) MES 2, (+) MES 3. (**a**), %: relative activity of ADH and trypsin, I_c and I_{ex}: light intensity in control and experimental cuvette, respectively, for bacteria bioassay. **a** and **b** are experiments with high and low biomasses of microalgae, respectively [25]

inoculated into the MES 1–3 and the bioassay reactions at the high biomass were recorded. In the second part of the experiment (Fig. 15b) MES 1–3 were reinoculated and the low biomass of microalgae was maintained in MES 1 ± 3.

In the course of the experiment microalgal species composition in the MES were recorded as varying greatly. Throughout the experiments, green filamentous algae *Ulotrix tenerrima* Kutz., *Ulotrix variabilis* Kutz., *Spirogyra varyans* (Hass.) Kutz., and *Spirogyra tenuissima* (Hass.) Kutz., dominated in MES 2 and 3. In MES 1 during the first 17 days of the experiment A the dominating species was the filamentous diatom *Fragillaria virescens* Ralfs., then the green filamentous algae *U. variabilis* became dominant. Generally speaking, in all the MES the plankton community of diatoms and green microalgae of quite diverse species composition was replaced by a simpler community of filamentous algae.

Response of the bioassays ranged from inhibition of bacterial luminescence and ADH and trypsin relative activity to stimulation of parameters of these bioassays (Fig. 15a, b). Parameters of the bioassay based on the coupled enzyme system remained essentially unchanged throughout the investigations (Fig. 16). Besides, the response of this bioassay to water samples taken from different MESs was the same (Fig. 16). There was no correlation between the response of the bioassays and the total algae biomass.

After 35 days of experiment B, benzoquinone 10 mg $\times$ L^{-1} was added in MES 1. Benzoquinone was taken as a xenobiotic for two reasons. First, quinones are greatly responsible for the dissemination and destruction of the water stretches and their inhabitants [57]. Second, Kudryasheva et al. [58, 59] have shown that the bioluminescent system L + R is a specific assay for quinones. The concentration chosen was close to benzoquinone content in the wastewater of the paper industry and higher than LC_{50} for luminous bacteria *Photobacterium phosphoreum* 10 μg $\times$ L^{-1} [16]. When benzoquinone was added to MES 1, parameters of all bioassays changed drastically. The response of the bioassay based on the coupled system was a decrease of luminescence intensity, an increase in the time needed to reach the maximum, and the occurrence of the induction period on the first day of intoxication (Fig. 16). The luminous bacteria-based bioassay showed a complete absence of light ($I_{ex} = 0$) from the water from the microecosystem where the toxicant was added (Fig. 15b). The bioassay based on ADH and trypsin showed a decrease in the activity of the enzymes when the bioassay was performed on the sample where benzoquinone was added (Fig. 15b).

2.7.2 Bioluminescent Control for Blooming Water

"Bloom" in the water body can also change the response of the bioassays. It was shown earlier that bloom of blue-green algae was the characteristic feature of the ecosystem which indicated the distinctive rate and kinetics of the phenol biodegradation [60]. No bloom of blue-green algae was observed in the water body under investigation, thus we used a laboratory culture of blue-green microalgae *Spirulina platensis* as a model of a blooming pond.

Dynamics of bioassay data and growth of algae are depicted in Fig. 17. One can see that throughout the algal growth period, the response of the bioassay based on the coupled enzyme system varied insignificantly. It is evident from the assay data

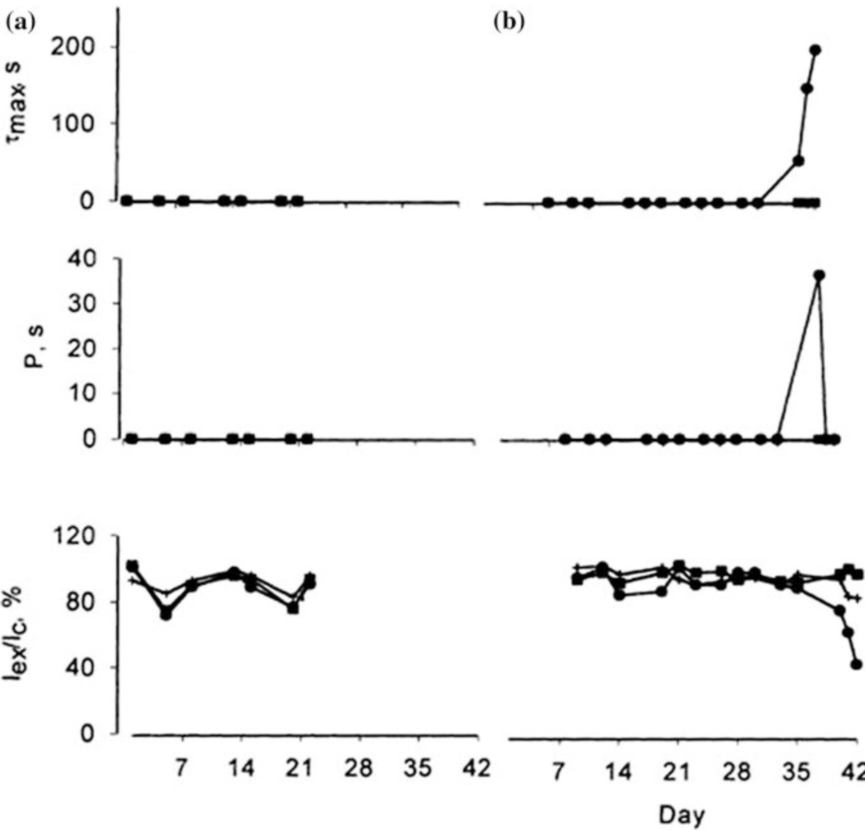


Fig. 16 Dynamics of parameters of bioassay based on coupled enzyme system NADH:FMN-oxidoreductase-luciferase for MES: (●) MES 1 (treatment with benzoquinone on the 35th day of experiment (**b**), (■) MES 2, (+) MES 3. **a** and **b** are experiments with high and low biomasses of microalgae, respectively, τ_{max} time of reaching the light maximum, *P* induction period, I_c and I_{ex} light intensity in control and experimental cuvette, respectively [25]

based on the triple enzyme systems that during the first days of their growth algae release metabolites inhibiting ADH and enhancing trypsin activity. After 4 days of algal growth, trypsin activity in the presence of the culture medium did not change. Response of the bioassays to the algal culture medium was the same in each of the three replicates. The bioassay based on ADH was the most sensitive. By the eighth and ninth days, the inhibition of the triple enzyme system with ADH was almost full (Fig. 17).

Therefore, bioluminescent assays show that their sensitivity differs by water quality, even in the case of the unpolluted pond. The assay based on luminous bacteria proved to be the most sensitive. The difference of water quality between the epilimnion and hypolimnion were due to natural causes rather than by any pollution. Hence, the result of this assay is determined by the depth at which water

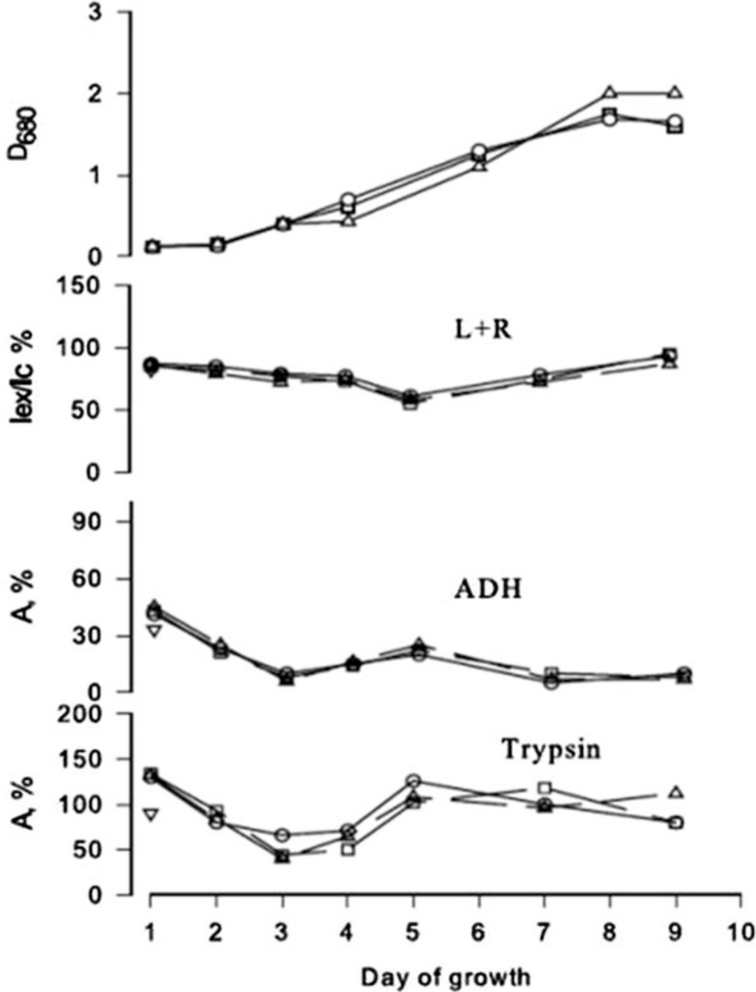


Fig. 17 The growth of microalgae biomass and the bioluminescent bioassay parameters for the study of the culture medium of *S. platensis*. (∇) effect of culture growth medium at the beginning of experiment, (○, □, Δ) three replicates, D_{680} optical density of *S. platensis* culture medium at 680 nm (the maximum of chlorophyll), *A* relative activity of ADH and trypsin, I_c and I_{ex} light intensity in control and experimental cuvette, respectively, for NADH:FMN-oxidoreductase-luciferase bioassay [25]

samples were taken (i.e., presence of the water from deoxygenated hypolimnion); this should be remembered when using this assay for the monitoring of water bodies.

Changes in the response of bioassays over the period of investigation were not related to pollution of the water body. Parameters of the bioassays did not change dramatically even in the periods of bloom of the green algae *V. aureus*. Indeed, in

contrast to blue-greens, bloom of green algae is not toxic. In addition, there is no correlation between a change in parameters of the bioassays and any of the pond ecosystem components (pH, dissolved oxygen, NO_2, NO_3, NH_4, etc.). From our data we could not determine the factor responsible for variations of parameters of the bioassays over the season. Apparently, such fluctuations in bioassay parameters should be regarded as natural for the unpolluted water body and must be accounted for by natural variability of water quality in the pond ecosystem.

It is evident from the experiments with the blue-green algae *S. platensis* that in the course of their growth these algae release substances that have the strongest effect on the ADH activity. Two components of the algal culture medium could change the response of the bioassays. They are culture medium mineral salts and metabolites released by algae as a result of their vital activity. Mineral salts are consumed by the growing algae, so their contribution to the inhibition of the enzyme activity decreases. The decrease of the ADH activity may take place due to an accumulation of metabolites in the medium at the end of the exponential growth phase.

There was no response of the coupled enzyme system, L + R, to the laboratory culture of the blue-green algae (Fig. 17). Meanwhile, the bioassay with trypsin responded to the algae medium at the early exponential stage of growth (the first 4 days). At this stage, the algae may release protease inhibitors into the culture medium.

Model experiments have shown that contamination of the MES with xenobiotics leads to sharp changes in parameters of all the bioassays. Here again, the most sensitive assay was that based on luminous bacteria. The most likely reason for this must be that xenobiotics (benzoquinone) affect a number of parameters of bacterial vital activity. They can affect respiratory and other oxidation-reduction processes in the cell, as well as disrupt membrane structures of the cell. As a result, the cells die, and do not produce the light.

The assay based on the coupled enzyme system is specific for quinones. If the water body is contaminated with quinones, a number of parameters of this assay change: P, T_{max}, intensity. The induction period appeared because the NADH was oxidized by benzoquinone. After the period of quinone reduction (P), by Reaction 2, FMN can be reduced, and further stages of the bioluminescent reaction with emission of a quantum of light become possible [59]. The ADH activity decrease may also be explained by the sequence of the redox NADH-dependent processes which may occur in the triple enzyme system NADH:FMN-oxidoreductase-luciferase-alcohol dehydrogenase [50].

The decrease of trypsin-relative activity may be explained by protein modification, for example, oxidation of the functional groups of amino acids contained in luciferase and trypsin (SH, etc.).

Thus, it has been shown that for the unpolluted water body fluctuations in the bioassay parameters were insignificant and resulted from natural variability of the pond ecosystem. Parameters of the assay changed sharply when the water body was contaminated with xenobiotics and in the case of bloom of blue-green algae. It is necessary to emphasize that ranges of variability of bioassays, which occurred in

the unpolluted pond and unpolluted MESs, were significantly lower than the degree of bioassay response after the addition of the pollutant (benzoquinone). Thereby we could detect the effect of pollutants such as quinones, within the variability of responses, caused by natural water.

Sensitivity of the assays to contaminants of polluted or “blooming” pond has been shown to differ, inasmuch as the mechanisms of xenobiotic (benzoquinone) action on the assay systems are different. Hence, the data of a single assay cannot provide a basis for a conclusion about the presence or absence of toxic substances in a water body. Only a set of assays, such as the one used in the present study can be applied as an alarm system to detect an acute toxicity of aquatic ecosystems.

2.8 Signal System of Enzymatic Assays for Toxicological Bioassay of Pesticides

Pesticides vary in their toxicity mechanism and character; for example, they can be carcinogenic or mutagenic, or they can affect the respiratory, endocrine, immune, or nervous systems [61, 62]. The effect of pesticides (organophosphates and pyrethroid preparations) on the bioluminescence of the four systems has been analyzed. The characteristics of the toxic substances analyzed are listed in Table 7.

Four bioluminescence assay systems were used in our studies (Table 8). For each of the analyzed substances, values of EC_{50} and EC_{20} were obtained (Table 8). They constituted 50 and 20 % of the loss of luminescence for the coupled enzyme system and luminous bacteria, or 50 and 20 % loss of the relative enzymatic activity for the triple enzyme systems with ADH and trypsin. The parameter EC_{20} was taken for the authentic determination of a toxicant’s impact on bioluminescence and for the comparison of the toxicant maximum permissible concentration.

The sensitivity of the coupled enzyme system to the impact of organophosphorous compounds differed widely. The EC_{20} of pirimiphos-methyl (Table 8, No. 2), for instance, was 0.9 mg $\times$ L^{-1}, whereas glyphosate (Table 8, No. 3) had no effect at all on the bioluminescence of the coupled enzyme system. It should be noted that glyphosate is a herbicide that has low toxicity to animals. Hence, their toxicity is in good correlation with bioluminescence inhibition.

The triple enzyme systems with ADH and trypsin are more sensitive to organophosphorous compounds (Table 8). The sensitivity of luminous bacteria to the impact of organophosphorous compounds is comparable to that of the coupled enzyme system (Table 8). For example, the EC_{20} of pirimiphos-methyl is the same in both cases, 0.9 mg $\times$ L^{-1}.

All the studied test systems were very sensitive to pyrethroid substances in the range 0.1–3 mg $\times$ L^{-1} (Table 7, Nos. 4–7). These insecticides, synthetic analogues of natural pyrethrins, act through intestinal contact, thereby affecting the nervous and the immune systems [63–65]. With pyrethroid, the values of EC_{20} are smaller for the bacterial system than for the coupled enzymatic system. To

Table 7 Characteristics of toxic substances analyzed by bioluminescence assays [51]

Number	Substance	Structural formula	Mr	Type of Substance
1.	Malathion		330.36	O
2.	Pirimiphos-methyl		305.33	O
3.	Glyphosate		169.07	O
4.	α-Cypermethrin		416.30	P
5.	Bifenthrin		422.87	P
6.	Deltamethrin		505.2	P
7.	λ-Cyhalothrin		449.85	P

compare: with deltamethrin (Table 8, No. 6) the values of EC_{20} are 0.06 and 0.001 mg $\times$ L^{-1} for the coupled enzyme system and luminous bacteria, respectively. This is attributed to permeability and destruction of cell membranes due to the high lipophilicity of these substances. Sensitivity of the triple enzyme systems to certain substances is similar to that of luminous bacteria.

Pyrethroid and organophosphorous compounds inhibited trypsin activity. Organophosphorous compounds are known to have an effect on proteolytic enzymes [61, 66]. They are able to inhibit esterase and proteolytic activity of both trypsin and chemotrypsin. This may account for the inhibitory effect of organophosphorous compounds observed in the triple enzyme system with trypsin.

Table 8 The influence of toxic substances on bioluminescent assay systems [51]

Number	Substance	Luminous bacteria photobacterium phosphoreum		Coupled enzyme system		Triple enzyme system with trypsin		Triple enzyme system with ADH	MPC (mg L^{-1})	PDD (mg kg^{-1})
		EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{20} (mg L^{-1})		
1.	Malathion	7	3.7	4	1.1	0.25	0.13	0.4	0.05	0.02
2.	Pirimiphos-methyl	5	0.9	3	0.9	7	3.3	11	0.01	0.01
3.	Glyphosate	40	30	Not measurable	>40	Not measurable	>40	>40	0.02	0.1
4.	α-Cypermethrin	0.5	0.31	0.7	0.3	1.61	0.16	10	0.002	0.01
5.	Bifenthrin	0.15	0.09	1.25	0.3	1.17	0.11	5	0.005	–
6.	Deltamethrin	0.01	0.001	0.5	0.06	1.3	0.32	0.003	0.006	0.01
7.	λ-Cyhalothrin	0.38	0.08	10	2.5	1.35	0.08	0.05	0.001	0.002

MPC maximum permissible concentration; *PDD* the person's permissible daily dose of a toxic substance (mg kg^{-1} body weight)

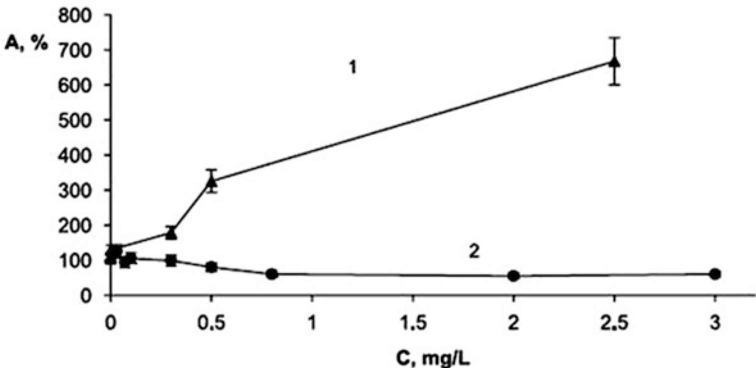


Fig. 18 Relative activity (*A*) of ADH (*1*) and trypsin (*2*) under different concentrations of deltamethrin. Influence of the pollutant on triple enzyme systems resulted in trypsin inhibition and ADH activation [51]

The triple enzyme bioluminescence systems included enzymes of different classes: ADH is an oxidoreductase and trypsin is a proteolytic enzyme. Therefore, the sensitivities and ways of interaction of ADH and trypsin with pollutants are different. All the toxic compounds studied, except malathion (Table 7, No. 1), had a weak stimulatory effect on the triple enzyme system with ADH. Figure 18 shows that deltamethrin inhibited the triple enzyme system with trypsin and activated the triple enzyme system with ADH.

Inhibition or activation of enzymatic activity by more than 20 % is indicative of the presence of toxic substances in the analyzed samples. Pesticides differ in their toxicity to animals [62, 65, 67, 68]. There are potent pesticides ($LD_{50} < 50$ mg × kg^{-1} animal's body weight) and pesticides of high (50–200 mg × kg^{-1}), medium (200–1,000 mg × kg^{-1}) and low (more than 1,000 mg × kg^{-1}) toxicity. Organophosphorous pesticides, with few exceptions, are of high and medium toxicity, but are rather quickly inactivated in the environment.

Table 8 gives the person's permissible daily dose (PDD) of a toxic substance (mg × kg^{-1} body weight) and the MPCs (the established hygienic standards of the Russian Federation: N. 1.2.1323-03). The values of EC_{20} for the studied bioluminescence systems are much higher than the MPCs. This means that the bioluminescence systems are not sensitive enough to detect a toxic substance at its MPC; however, it will allow the avoidance of a false signal given by background fluctuation of water quality, and allow authentic reaction with toxicants present in the water with concentrations exceeding MPCs, which are indeed dangerous for living organisms.

Considering EC_{20} and PDD (based on an average man's weight of 70 kg), these values are similar. By way of example, the PDD for malathion (Table 7, No. 1), corrected for a man's weight, is 1.4 mg × kg^{-1}, whereas the EC_{20} of the coupled enzyme system is 1.1 mg × L^{-1}. This shows that sensitivity of bioluminescence systems can be used to determine doses toxic to humans.

For pesticides, the sensitivities of the bioluminescence test systems were compared to those determined by other bioassays [62, 67, 69–72]. In some cases, bioluminescence bioassays are more sensitive. For example, the sensitivity of mice to deltamethrin (Table 7, No. 6), as established by the most sensitive (for mammals) hematological test, the erythrocyte micronuclei assay, is 90 mg $\times$ kg^{-1} [70], whereas the sensitivity of the bioluminescence test based on luminous bacteria is 0.001 mg $\times$ L^{-1}. The highest sensitivity reported was that of fish used in bioassays [62, 70]. However, analysis of that kind lacks fast response (the time required for testing is 7 days) and is very laborious.

Thus, bioluminescence tests are sufficiently sensitive to detect compounds toxic to humans and other mammals. The results presented can provide the basis for the development of an alarm test bioluminescence system based on either intact bacteria or any of several coupled enzyme systems. The described biotesting methods are proposed for measurements in the environment presumably contaminated with toxic agents as a result of industrial pollution.

Inasmuch as test systems differ in their sensitivities to various toxic agents, a deviation from the background in even one of the tests signals the presence of a "toxic factor" in the water. In this situation, chemical analysis is recommended.

3 Summary

It is necessity to point out that in vitro bioluminescent assay is a promising method for analyzing the integral characteristics of various media, and its potential hasn't been used up yet. For example, the coupled enzyme system is used for monitoring radiation toxicity, keeping track of the radiation effect on a microbiological and biochemical level. The bioluminescent method of radiation toxicity monitoring in solutions containing alpha- and beta-emitting radionuclides has been developed [73, 74].

A new trend in using bioluminescent methods is the efficiency assessment of detoxification by natural humic substances. This method is based on the quantitative determination of the antioxidant activity of natural detoxicants, humic substances. This method makes it possible to assess their remediation properties which are revealed by reducing the effect of toxic substances on the luminous intensity of bacteria bioluminescent reaction in the presence of humic acids [75, 76].

Moreover, the application area of bioluminescent enzymatic assays based on the estimation of integral toxicity is not limited by ecology. Methods based on the use of enzymatic bioluminescent bacterial systems are applied for food product quality. A method was suggested for detection of L- and D-lactate in beer [77]. The effects of the mycotoxins produced by fungi of the genus Fusarium on the coupled enzyme system: NADH:FMN-oxidoreductase-luciferase were studied [78–80]. A possibility was demonstrated to use bioluminescent methods for safety evaluation of food preservatives. Mei and coauthors [81] suggested detecting living bacterial

cells according to detection of cellular NADH using the coupled enzyme system: NADH:FMN-oxidoreductase-luciferase.

Integrated bioluminescent methods are also very promising for medical research, for example, for evaluating the gravity of endotoxicosis during treatment in surgery and therapy. The methods are highly sensitive, rapid, and simple and allow quantitative determination of the degree of seriousness of illness and the estimation of the severity of a patient's condition, disease course prediction, estimation of the efficiency of the used detoxification methods, and determination of the optimal operation mode of drainage facilities made of semipermeable membranes [82–84]. Controlled perfusion of rats' liver demonstrated a possibility of using an integrated bioluminescent method for the intensity assessment of pathologic oxidation processes [85]. A very interesting and promising trend in the development of integrated bioluminescent testing is the creation of rapid analysis for the assessment of human organism reaction to physical and mental stress. Analysis is made by comparing the light emission intensity of the coupled enzyme system NADH:FMN-oxidoreductase-luciferase in the presence of a person's saliva taken before and after a certain stress load [86]. The main advantages of the bioluminescent method are not only its rapidity, high precision, and sensitivity, but also noninvasiveness, because human saliva is analyzed, which reflects the functional state of a person just as blood does.

Thus, the new approach of biotechnological design developed on bioluminescent enzymatic biosensors, methods, and reagents has been described in the chapter. To solve the problem of how to detect, identify, and measure the contents of the numerous chemical compounds in environmental monitoring, food product monitoring, and medical diagnostics, we proposed Bioluminescent Enzyme System Technology BEST™, wherein the bacterial coupled enzyme system: NADH:FMN-oxidoreductase-luciferase substitutes for living organisms. BEST™ was introduced to facilitate and accelerate the development of cost-competitive enzymatic systems for use in biosensors.

A patented stabilization and immobilization process produces the multicomponent reagent Enzymolum, which contains the bacterial luciferase, NADH:FMN-oxidoreductase, and their substrates, coimmobilized in starch or gelatin gel. The reagent is currently produced in tablet form and can be used only in the cuvette variant of a bioluminometer. The other forms, for example, on the plane table, strips, and others were also introduced for bioluminescent analysis. Enzymolum can be integrated as a biological module into the portable biodetector–biosensor of original construction.

Enzymolum is the central part of the Portable Laboratory for Toxicity Detection (PLTD), which consists of a biological module, a biodetector module, a sampling module, a sample preparation module, and a reagent module. PLTD allows us (a) to detect a wide range of toxic substances, more than 25,000 compounds; (b) to perform rapid screening for toxicity in emergency situations in the field and laboratories; (c) to develop systems for analyzing individual compounds; (d) to develop systems to evaluate the degree of overall toxicity; (e) to keep the high sensitivity of reagents for many years; and (f) to perform biotesting in the presence

of high concentrations of organic substances in water. The last item suggests the uniqueness of enzymatic bioassays, because no bioassays based on living organisms give consistent results under such conditions. Living organisms use organic substances for food and thus bioassays based on living organisms do not reflect environmental quality correctly, resulting in the absence of accuracy and reliability of analysis.

Prototype biosensors developed with this technology offer cost advantages, versatility, high sensitivity (up to 10^{-14} mol of analyte), rapid response time (less than 3 min), extended shelf life (up to 2 year), and flexible storage conditions (up to +25 °C).

The enzyme biotesting approach was used as a platform technology to certify "Method to measure the intensity of bioluminescence with the help of the 'Enzymolum' reagent to detect the toxicity of drinking, natural, waste, and treated waste water" [48]. The laboratory will be the principal example of a whole family of new, portable, professional laboratories for ecological monitoring, food quality laboratories, military departments, and other monitoring, teaching, security, and research organizations.

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Detection of Organic Compounds with Whole-Cell Bioluminescent Bioassays

Tingting Xu, Dan Close, Abby Smartt, Steven Ripp and Gary Sayler

Abstract Natural and manmade organic chemicals are widely deposited across a diverse range of ecosystems including air, surface water, groundwater, wastewater, soil, sediment, and marine environments. Some organic compounds, despite their industrial values, are toxic to living organisms and pose significant health risks to humans and wildlife. Detection and monitoring of these organic pollutants in environmental matrices therefore is of great interest and need for remediation and health risk assessment. Although these detections have traditionally been performed using analytical chemical approaches that offer highly sensitive and specific identification of target compounds, these methods require specialized equipment and trained operators, and fail to describe potential bioavailable effects on living organisms. Alternatively, the integration of bioluminescent systems into whole-cell bioreporters presents a new capacity for organic compound detection. These bioreporters are constructed by incorporating reporter genes into catabolic or signaling pathways that are present within living cells and emit a bioluminescent signal that can be detected upon exposure to target chemicals. Although relatively less specific compared to analytical methods, bioluminescent bioassays are more cost-effective, more rapid, can be scaled to higher throughput, and can be

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designed to report not only the presence but also the bioavailability of target substances. This chapter reviews available bacterial and eukaryotic whole-cell bioreporters for sensing organic pollutants and their applications in a variety of sample matrices.

Keywords Bacterial luciferase · Bioavailability · Bioreporter · Bioluminescence · BTEX · Dioxin · Endocrine disruptors · Environmental monitoring · Firefly luciferase · Hydrocarbon · PAH · PCB

Abbreviations

AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
ARE	Androgen response element
ARNT	AhR nuclear translocator
BPA	Bisphenol-A
BTEX	Benzene, toluene, ethylbenzene, and xylene
CALUX	Chemical-activated luciferase expression
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DRE	Dioxin-responsive element
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EE2	17α-ethynylestradiol
ER	Estrogen receptor
ERE	Estrogen response element
GC	Gas chromatography
GR	Glucocorticoid receptor
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PMT	Photomultiplier tube
PR	Progesterone receptor
T3	3,3′,5-triiodo-L-thyronine
TCA	1,1,1 trichloroethane
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCE	Trichloroethylene
TR	Thyroid receptor

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1 Introduction

Synthetic organic chemistry started in the nineteenth century when, instead of extracting urea from an animal kidney, the German chemist Friedrich Wöhler produced it from inorganic substances, and French chemist Marcellin Berthelot chemically synthesized fatty acids that are not found in nature [1]. Over the ensuing 200 years, chemists have subsequently learned to create an enormous number of organic compounds, both of natural and synthetic origin, that have become essential input materials for consumer products, agriculture, manufacturing, pharmaceutical, and life science industries, and myriad other applications. However, despite their industrial value, some of these compounds have unfortunately become associated with adverse health effects in humans and animals. For instance, exposure to benzene, a naturally occurring aromatic hydrocarbon found in crude oil, has now been linked to decreased circulating blood cell counts, leukemia, and immunotoxicity [2], as has exposure to the toxic synthetic compound dichlorodiphenyltrichloroethane (DDT), which was used extensively as an insecticide until it was banned from agricultural use worldwide because of its toxicity toward a wide range of organisms. These compounds, like many other organic pollutants, are persistent and prone to bioaccumulation, endowing them with a large potential to negatively affect the well-being of living organisms.

The increasingly large scale of industrial chemical production, and its corresponding increase in anthropogenic chemical consumption, has driven up a demand for effective tools and approaches that can both detect the environmental occurrence of these compounds as well as assess their potential biological effects following exposure. These environmental monitoring efforts have traditionally employed analytical methods such as high-performance liquid chromatography

(HPLC) and gas chromatography coupled with mass spectrometry (GC/MS) to detect and quantify toxic chemicals, and the ability of these analytical methods specifically to measure chemical concentrations at high sensitivities has been instrumental for the evaluation of the level of contamination. However, these methods have proved to be time-consuming, expensive, and relatively difficult to perform and, more important, are not capable of measuring bioavailability and biological impact, two important aspects of the risk assessment paradigm.

To overcome these shortcomings, biological assays using living whole-cell bioreporters have been developed to provide more biologically relevant information. Bioluminescent bioreporters in particular, due to their ability to generate an easily measurable light signal, have been well validated in the laboratory and extensively applied in environmental monitoring. These bioreporters, being genetically transformed to express the luciferase genes of bacterial origin (*luxAB*), the full bacterial bioluminescent system (*luxCDABE*), or the firefly luciferase gene (*luc*) as a means of light production, provide a rapid, simple, and cost-effective complement to analytical chemical methods. As living entities, whole-cell bioreporters act as proxies for humans and other organisms to prewarn the occurrence of potentially toxic substances. Most environmental applications have traditionally employed bacterial-based bioreporters for this purpose, however, eukaryotic cell-based bioreporters are increasingly being utilized to provide more human- and animal-relevant data. This chapter reviews the development of both bacterial and eukaryotic cell-based bioluminescent bioreporters for sensing a broad range of organic compounds and provides an overview of the utility and limitations of these bioluminescent bioassays in practical applications.

2 Detection of Organic Compounds Using Bacterial Bioluminescent Assays

Being genetically easy to manipulate and displaying rapid and robust growth, bacteria have been extensively employed as hosts for bioreporter development. Despite the toxic nature of organic pollutants, evolution has provided some bacteria with unique genetic properties that allow them to adapt to the presence of toxic chemicals by utilizing them as carbon sources. As the generation of proteins involved in the degradation of exposed pollutants is an energy-consuming process and costs fitness under unexposed conditions, the catabolic process is carefully regulated at the transcriptional level in such a way that it is only initiated upon exposure to its corresponding chemical input. To exploit this unique feature, bacterial bioluminescent bioreporters are constructed by transcriptionally integrating reporter genes (*luxAB*, *luxCDAB*E, or *luc*) within special catabolic pathways that specifically respond to the presence of target compounds. Fortunately, the diversity of catabolic capabilities and sophisticated characterization of responsible genetic components has provided a sizable toolbox for bioreporter

construction. This section provides an overview of the genetic foundations for bacterial bioluminescent bioreporter development and their applications toward detection of a variety of common organic contaminants (Table 1).

2.1 Benzene, Toluene, Ethylbenzene, and Xylene

The monocyclic hydrocarbons benzene, toluene, ethylbenzene, and xylene (BTEX) are found across various environmental matrices such as water, soil, and other sediments due to contamination with petroleum products resulting from gasoline spills, underground storage container leaks, runoff from manufacturing plants, and so on. Traditionally, a sample suspected of BTEX contamination would be subjected to a lengthy extraction process to purify the available chemicals, followed by analytical-based testing to identify the compounds present. In an effort to ease the process of detecting and identifying chemical pollutants, bioluminescent reporter strains have been developed that modulate signal production in response to BTEX chemical exposure, indicating their presence in a sample. To accommodate the disparate needs of detection, a variety of reporter constructs has been developed over the years, ranging from the straightforward introduction of bioluminescent genes into naturally BTEX-degrading organisms to track their prevalence, to more complex introductions of both bioluminescent and BTEX-degrading genes into specialized reporter organisms that can be tailored to the needs of a particular investigation. Although this section focuses only on bioluminescent BTEX reporter organisms that modulate signal in response to compound detection, it should be noted that there is a variety of additional sources that have evaluated BTEX presence and toxicity using constitutively bioluminescent reporters as well [3–5].

Due to the prevalence of BTEX in the environment, a multitude of bioluminescent bioreporters have been developed across a variety of different host strains. The majority of these bioreporters function by leveraging the expression of the TOL plasmid degradation genes that were originally identified in *Pseudomonas putida* mt-2 [6]. These genes function across two distinct pathways consisting of an upper pathway and a *meta* pathway. In this system, BTEX compounds are first oxidized in the upper pathway, and then proceed to the *meta*-cleavage pathway where they are further broken down before ultimately being routed to the Krebs cycle [7–9]. Governing the expression of these pathways are two regulators, of which the primary XylR regulator is most often utilized for bioreporter design. This may seem counterintuitive because the *xylR* gene is constitutively expressed, but its resulting protein product remains inactive until physical interaction with a BTEX chemical imparts structural changes that permit it to bind to, and subsequently activate, the upper pathway promoter P_u [10]. Li et al. [11] were able to capitalize on this interaction and construct a pTOLLUX plasmid that utilized the *xylR* gene product to activate the transcription of an *Aliivibrio fischeri* (originally classified as *Vibrio fischeri* [12]) *luxCDABE* gene cassette that was fused to the

Table 1 Bacterial bioluminescent bioreporter for organic contaminants and representative environmental applications

Reporter strain	Reporter construct	Target compound	Detection limit	References	Environmental application
E. coli DH5α (pPROBE-LuxAB-TubT)	*tubT-lux*AB	BTEX	0.24 μM toluene	[14]	Simulated aquatic oil spill [14]
E. coli DH5α pGLTUR	*xylR*-P_u-*luc*	Benzene, Toluene, Xylene	10–20 μM toluene 3 μM 3-xylene	[13]	Soil [13, 131]
P. putida TVA8	*tod-luxCDABE*	BTEX TCE	0.03–50 ppm BTEX 1 μM TCE	[15]	Soil [3] Water [18]
E. coli HMS174 (pOS25)	*ipbR-luxCDABE*	Hydrophobic compounds	1 μM toluene 0.1 μM naphthalene	[22]	Soil [132]
E. coli DH5α pTOLLUX	*pR/xlyR*-P_u-*luxCDABE*	BTEX	N/A	[11]	Soil and groundwater [11]
B. sartisoli RP007 (pPROBE-phn-luxAB)	*phnS-luxAB*	PAH	0.17 μM naphthalene	[14]	Simulated aquatic oil spill [14]
P. putida pPG7-JAMA21	*nahR*-P_{sal}-*luxAB*	Naphthalene	0.50 μM naphthalene vapor	[133]	N/A[a]
P. fluorescens HK44 (pUTK21)	*nahR*-P_{nahG}-*luxCDABE*	PAH	12–120 μM	[29]	Soil [32, 131, 134]
E. coli DH5 (pGEc74, pJAMA7)	*alkS*-P_{alkB}-*luxAB*	6–10-carbon alkane	25 nM octane	[37]	Groundwater [20] Soil [131]
E. coli	*alkBFG*, *alkJ-luxAB*	5–12-carbon alkane	10 μM octane	[39]	N/A
Acinetobacter baylyi ADP1	*alkR*-P_{alkM}-*luxCDABE*	Alkane	3 nM octane	[43]	Seawater [43]
E. coli	*alkR*-P_{alkM}-*luxAB*	Alkane	5 nM octane	[45]	N/A
P. putida F1G4	*sep-luxCDABE*	TCE	1 mM TCE	[50]	N/A
Methylobacterium extorquens	*dcmR*-P_{dcmA}-*luxCDABE*	DCM	1 μM DCM	[51]	N/A

(continued)

Table 1 (continued)

Reporter strain	Reporter construct	Target compound	Detection limit	References	Environmental application
Ralstonia eutropha ENV307 (pUTK60)	*bphR1-luxCDABE*	PCBs	0.80 μM 4-chlorobiphenyl 4.6 μM Aroclor	[53]	N/A
E. coli XL1-Blue	P_{pcbC}-*luxCDABE*	PCBs	0.1 mM biphenyl (lowest concentration tested)	[56]	N/A
E. coli XL1-Blue	P_{pcbC}-*luc*	PCBs	0.1 mM biphenyl (lowest concentration tested)	[56]	N/A
E. coli (pHYBP109)	*hbpR*-P_{hbpC}-*luxAB*	OH-PCBs	1 nM 2-hydroxy-3′,4′,5-trichlorobiphenyl	[58]	Simulated aquatic oil spill [14] Human serum [58]
P. fluorescens OS8(pDNdmpRlux)	*dmpR*-P_o-*luxCDABE*	Phenolic compound	0.30 μM 2-methylphenol 0.87 μM phenol	[63]	Groundwater and semicoke-dump leachates [63]
E. coli (pRLuc42R)	*luc*	Phenolic compound	0.5 μM phenol	[65]	N/A
Acinetobacter DF4-8	*mopR-luxCDABE*	Phenolic compound	0.03 mM phenol	[67]	N/A

[a] N/A not available

native P_u promoter. When expressed in *Escherichia coli* DH5α, and assayed in 96-well microtiter plates, the resulting bioluminescence could be detected after a 2-h incubation with 7.5 μM toluene. However, because the XylR regulator can be activated by a variety of BTEX chemicals, a test with known concentration toluene spikes is required in parallel with all environmental samples. It was determined that, under these conditions, the reporter was capable of detecting a concentration of 168 μM of nonspecific BTEX compounds in soil, and 362 μM of nonspecific BTEX compounds in groundwater. However, additional testing is still required to determine which specific compound(s) are present.

In an alternative but somewhat similar approach, Willardson et al. [13] developed a BTEX reporter strain by employing the XylR regulator to govern expression of the firefly luciferase gene (*luc*). The resulting *xyl*R-P_u-*luc* fusion was housed on the pGLUTR plasmid and expressed in *E. coli* DH5α. The resulting reporter was then used to detect BTEX compounds in both soil and water samples [13]. Notably, the water samples used in these experiments were taken from near an underground storage tank known to have leaked BTEX compounds and were incubated directly with the reporter without preprocessing. Following this 1-h direct incubation, the samples were treated with luciferin substrate to induce signal generation and the total detectable BTEX concentration in the water sample was determined to be 215 μM of toluene equivalents. Unlike the water samples, this reporter still required soil samples to undergo an ethyl alcohol extraction to isolate any chemical pollutants prior to exposure. However, following this extraction and a subsequent dilution of the samples in medium, only a 1-h incubation was required before signal generation, and under these conditions 3.44 mM toluene equivalents could be detected. So although this method was still limited by its inability to report specific compounds, it was capable of detecting total BTEX compounds within 3 % of conventional detection methods, making it a powerful tool for general BTEX detection.

In addition to reporters that utilize the TOL plasmid, a second class of reporter organism has been developed around the toluene benzene utilization pathway (*tbu*) from *Ralstonia pickettii* PKO1. Similar to the TOL-encoded pathway, the *tbu* pathway is regulated by the TbuT protein, which activates the *PtubA1* promoter in the presence of a BTEX inducer compound. Building upon this pathway, Tecon et al. [14] developed an *E. coli*-based bioreporter that harbors a pPROBE-LuxAB-TbuT plasmid whereby the TbuT regulator acts on the *PtubA1* promoter when a BTEX inducer compound is present to transcribe the *luxAB* genes from *A. fischeri*. Because only the *luxA*B genes were present, and not the full *luxCDABE* operon, all assays using this reporter required the addition of the n-decanal substrate prior to bioluminescent production. To determine its functionality under environmentally relevant conditions, this bioreporter was used to monitor an artificial oil spill. Using a 96-well microtiter plate assay, this bioreporter detected 4–12-μM toluene equivalent concentrations in contaminated seawater samples that were collected as early as 6 h after the spill. However, similar to the TOL-based systems, the inability to differentiate between BTEX compounds limits this reporter to only broad-spectrum compound detection.

The third class of reporters is typified by the bioluminescent *P. putida* TVA8 BTEX bioreporter developed by Applegate et al. [15]. This reporter, as do all members of its class, utilizes the *P. putida* T-2 toluene degradation pathway (*tod*) that was originally characterized by Zylstra et al. [16] and Wang et al. [17]. Similar to the previously described pathways, the *tod* pathway consists of a series of genes responsible for BTEX oxidation that are under the control of a BTEX regulatable promoter. Using this system, *P. putida* TVA8 was constructed by transposon insertion of the bioluminescent *luxCDABE* gene cassette downstream of the *todX* and *todR* genes for toluene recognition and transcriptional activation and validated in wastewater samples for its ability to detect BTEX compounds [18]. During the experiment, *P. putida* TVA8 was inoculated with a 1:10 ratio of wastewater and sampled every 30 min for 8 h to observe bioluminescence. Unlike the assays of Stiner and Halverson [19], no additional substrate was required because this construct contained the full *luxCDABE* cassette. Under these conditions, bioluminescence was detectable within the first 30 min [18] and significant bioluminescent responses were observed following treatment with 23 distinct compounds. Although this again highlights the inability of most BTEX bioreporter organisms to differentiate individual BTEX compounds from one another, it also shows their utility as detection systems for organic pollutants in general. Following this successful validation, the same reporter was later utilized to test groundwater located beneath an airfield site [20], where it successfully differentiated between contaminated and remediated sites as confirmed by analytical detection methods.

The final pathway that has been used for BTEX bioreporter construction is the isopropylbenzene (*ipb*) pathway. This pathway, which was originally identified in *P. putida* RE204 [21], consists of a regulatory protein (encoded by *ipbR*), an operator/promoter *ipbo/p*, and the isopropylbenzene dioxygenase gene (*ipbA*). To develop a BTEX bioreporter using this system, Selifonova and Eaton [22] transformed an *E. coli* HMS174 strain with a plasmid containing the *ipb* genes fused with the *lux* gene cassette of *A. fischeri*. This construct was then used to detect aromatic compounds in hydrocarbon mixtures (jet fuel, diesel fuel, and creosote) and for the direct detection of hydrocarbons extracted from sediments. It was demonstrated that the reporter could detect a concentration as low as 0.01 ppm in creosote hydrocarbon mixtures. Because the sediments contained a mixture of hydrocarbons in varying quantities, light detection was measured from dilutions of the total extracted hydrocarbons to determine if bioluminescent production increased corresponding to the increase of hydrocarbons. It was demonstrated that the reporter did produce bioluminescence corresponding to the sediment extraction dilutions and could detect hydrocarbons in a dilution as low as 1:500. However, because this reporter is not specific to BTEX compounds, the amount of light produced may not indicate the true concentration of BTEX compounds in mixed samples.

As a whole, it can be said that the varied classes of BTEX reporters have successfully met their goal of providing a faster and lower cost method for the detection of BTEX compounds. However, the major caveat for these reporters is

their inability to differentiate individual BTEX compounds. Because each bioreporter was constructed to detect a number of compounds that are under the BTEX classification, pinpointing which contaminant(s) are present in the sample is not currently possible. Therefore, in order to determine the exact chemical contaminant further analysis of a sample still needs to be done. Despite this drawback, these reporters provide a simple and efficient method for the rapid screening of multiple categories of environmental samples, making them a valuable first-line analysis tool for large-scale monitoring projects.

2.2 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are compounds consisting of two or more fused benzene rings. Due to repeated spills and the seepage of petroleum products, they have become some of the most common soil and water contaminants and can be found around the globe. There is a pressing need for the identification and remediation of PAHs because they pose significant human health risks such as heart disease, cancers, and kidney and liver damage [23]. In order to meet this need, PAH-detecting bioreporters have been developed that utilize either the naphthalene, phenanthrene, or isopropylbenzene degradation pathways for control of their bioluminescent signals.

By far, the majority of the PAH bioreporters exploit the naphthalene degradation pathway in order to modulate their bioluminescent signal. This pathway consists of two separate systems, an upper pathway (*nahABCDEF*) that degrades naphthalene to salicylate, and a lower pathway that then degrades the salicylate to acetaldehyde and pyruvate [24]. A single regulatory gene, *nahR*, governs the expression of these genes in response to naphthalene, making it an ideal target for bioreporter design. Burlage et al. [25] were the first to make use of this pathway when they constructed *P. putida* RB1351. This strain contains the *luxCDABE* gene cassette under the control of the upper NAH promoter, P_{nah}, allowing bioluminescent expression to be modulated in response to naphthalene bioavailability. This reporter strain has been extensively investigated by Dorn et al., who refer to the strain as *P. putida* RB1353 [26–28]. From these studies, it has been concluded that the *lux* expression can be altered from a 1 °C temperature change and a change in pH of 0.2 [27], the reporter can successfully be integrated into a fiber-optic detection system for monitoring microbial activity in porous media in real-time [28], and that the fiber-optic detection system can be used for the realtime in situ monitoring of bioactive zone formation and dynamics [26].

The most widely known naphthalene pathway-based reporter, however, is likely *P. fluorescens* HK44, which has become one of the most evaluated microbial bioreporters ever to be developed. Similar to *P. putida* RB1351, HK44 expresses the upper naphthalene pathway (*nahABCDEF*) and the regulator gene *nah*R, however, in the lower pathway the *nahG* gene is fused to the *luxCDABE* cassette from *A. fischeri* [29]. Using this set-up King et al. [29] demonstrated a

bioluminescent response to naphthalene at concentrations as low as 1.56 μM after 15 min of exposure in a chemostat culture. More important, however, it was also shown in this study that the bioluminescent signal generated by HK44 was capable of responding to naphthalene in a dose-responsive manner, allowing for realtime detection and monitoring. Throughout its widespread use, HK44 has since been tested against a variety of organic compounds and has been applied to monitor PAH occurrence in various environmental matrices (recently reviewed by Trögl et al. [30]). Although the majority of applications using strain HK44 have been performed on extracts of water, soil, and sediment samples, Valdman and Gutz [31] recently demonstrated the utility of agar gel-immobilized HK44 reporter cells for the detection of naphthalene and related compounds in the vapor phase. Various concentrations of naphthalene vapors were flowed into sampling tubes and bioluminescence was monitored in a luminometer. The limit of detection under this experimental design was determined to be 20 nM naphthalene, and a linear relationship between naphthalene concentration and bioluminescent response was obtained between the concentrations of 50 and 260 nM.

Because of the characteristics and popularity of the *P. fluorescens* HK44 bioreporter, it was selected as the model organism for a first of its kind multiyear controlled field release study. In 1996 *P. fluorescens* HK44 was released in a controlled environmental test site to monitor the long-term ability of a genetically modified organism to detect and degrade naphthalene. Over time, environmental naphthalene was detected in two ways, either through the detection of naphthalene vapors by HK44 biosensor modules interfaced with fiber-optic cables [32], or through direct interaction of HK44 bioreporter cells with naphthalene in the soil via observation of the resultant bioluminescent signal using a photomultiplier tube (PMT) [32]. The continued detection of HK44 throughout the 2-year study proved its ability to persist in the environment, and bioluminescent detection on-site demonstrated its utility as a continuous reporter for naphthalene bioavailability [32, 33].

It is important to remember, however, that despite the widespread use of *P. fluorescens* HK44 and its related naphthalene-based sensor organisms, other methods for PAH bioreporter construction have been employed. Among these has been the use of the phenanthrene degradative genes from the *Burkholderia* sp. strain RP007, which was first described by Laurie and Lloyd-Jones in 1999 [34]. Tecon et al. [126] exploited this operon for the construction of a bioreporter for the detection of naphthalene, phenanthrene, and related PAH compounds. The resultant reporter strain, *B. sartisoli* RP007, harnessed the regulatory genes *phnR* and *phnS* from the phenanthrene pathway to regulate expression of the *luxAB* genes. Under this design, when a PAH compound interacts with the PhnR protein it causes the downstream activation of the *phnS* promoter, which then allows transcription of the *A. fischeri luxAB* genes. Similar to other reporters that only contain the *luxAB* genes, this reporter is limited in that the substrate n-decanal must be added for the production of light concurrent with naphthalene exposure. However, when n-decanal is supplied, the reporter demonstrated a minimal detection limit of 0.17 μM after a 3-h incubation. When exposed to an artificial oil spill,

bioluminescence was detected above the minimal detection limit after 3 h, and continued to produce a response after 5 days, demonstrating a longevity of signal that can be crucial for environmental monitoring applications.

2.3 Alkane Aliphatic Hydrocarbons

Alkanes are saturated hydrocarbon structures that can be deposited environmentally from a wide variety of sources. Most commonly, however, they originate from the natural seepage of crude oil deposits or from anthropogenic releases of fuel products and industrial lubricants. Due to their highly hydrophobic nature, they are not acutely bioavailable, and therefore have traditionally been difficult to detect. Historically, the predominant method for alkane detection by microbial bioreporters has been through expression of the regulatory region of the alkane-responsive *alk* operon from *Pseudomonas oleovorans*. In 1973 it was discovered that the genes responsible for octane degradation in *P. oleovorans* were located on a plasmid and could be transferred between organisms [35]. Although it was known at this time that the degradation function encoded by these genes was inducible in the presence of alkanes, it took another 15 years before the genetic structure of the operon was fully identified [36]. It was then a further 9 years before it was first exploited as a sensing component for bioreporter development [37]. The *alk* operon consists of two distinct sections, with the first encoding three genes for alkane catabolism (*alkBFG*) and second encoding a regulatory component (*alkS*) that can activate transcription in the presence of 6- to 10-carbon alkanes [38].

Sticher et al. [37] were able to remove the *alkS* regulatory component and coexpress it along with a fusion of the inducible *alkB* promoter and the *Vibrio harveyi luxAB* genes. This effectively governed the transcription of the *luxAB* genes in response to alkane presence, although it required an exogenous application of decanal to serve as the substrate for the *luxAB* luciferase in order to elicit a bioluminescent response. Following a survey of decanal concentrations, it was determined that the assay conditions had to be amended with 2 mM decanal to ensure that the results were indicative solely of the induction of the *luxAB* genes by alkanes, and not limited by a lack of substrate for the resultant bioluminescent reaction. Under laboratory conditions, this decanal-supplemented assay was capable of detecting 5- to 10-carbon chain length alkanes with a response time of 1–2 h. The minimum detection level for octane was determined to be 24.5 nM, however, induction at this level resulted in only a 1.4-fold increase in light production. It is also important to note that the assay could be inhibited by the presence of alicyclic hydrocarbons, aromatic hydrocarbons, alkylbenzenes, or biphenyls, which limited its use in environmental applications. Despite this handicap, however, the assay was used to monitor diesel-oil–contaminated groundwater samples, but was limited to reporting in octane equivalents inasmuch as it is not specific for individual hydrocarbon species. Similarly, because of the

complex nature of diesel oil, a coassay was required to determine the level of inhibition caused by nonalkane chemicals, which then allowed a corrected octane equivalency to be determined. Although this assay format was not ideal, it represented the first time that a bioluminescent microbial assay was used to monitor for environmental alkane contamination, and provided a valuable first step toward the development of improved sensor moieties.

Building upon this system, Minak-Bernero et al. [39] were able to take further advantage of the remaining *P. oleovorans alk* operon genes and develop an alkane sensor that did not require the exogenous addition of decanal in order to produce a bioluminescent signal. To accomplish this, they constitutively co-expressed the *alkBFG* alkane catabolism genes, the *alkJ* alcohol dehydrogenase gene, and the *luxAB* genes. Under this system, the *alkBFG* gene products performed their native function of reducing the target alkanes to alcohols, the *alkJ* gene product then converted those alcohols into aldehydes, and the LuxAB luciferase proteins then used the resultant aldehydes as substrates for the generation of a bioluminescent signal. The ultimate result was a bioluminescent microbial bioreporter that could respond to the presence of alkanes within seconds after exposure to produce a detectable signal. This sensor was approximately as sensitive as the decanal-dependent sensor developed previously [37], giving a linear response to octane in the 10- to 200-μM range [39]. Additionally, because the *P. oleovorans alk* gene products were capable of modifying 5- to 12-carbon chain length primary alcohols and aldehydes [40], and the LuxAB luciferase protein could accept 6-carbon and longer chain length aldehydes [41], this sensor was theoretically capable of sensing any alkane between pentane and dodecane. However, because the system constitutively expressed all of the *alk* and *lux* genes, the sensor would report the detection of any bioavailable pathway intermediate products indiscriminately. This made it impossible to differentiate alkanes, alcohols, or aldehydes in a given sample. So although detection had become increasingly autonomous, the specificity of the system was reduced.

In 2010 there was a renewed interest in the detection of alkane hydrocarbons in seawater due to the highly publicized Deepwater Horizon oil spill in the Gulf of Mexico [42]. This spurred renewed testing with the available reporter strains and demonstrated that, although the detection characteristics were similar to those obtained in groundwater samples [37], the reporters could be inhibited by salt during in situ analysis with laboratory-contaminated seawater [14], limiting their use under environmental conditions. To overcome this deficiency, Zhang et al. [43] developed a bioluminescent reporter using the native *alk* operon system in *Acinetobacter baylyi* ADP1 rather than expressing a modified version of the *P. oleovorans alk* operon in *E. coli* as had been done previously. This reporter was constructed using homologous recombination to introduce the *luxCDABE* genes from *Photorhabdus luminescens* in place of the *alkM* alkane hydroxylase gene in the *A. baylyi* ADP1 chromosome, placing them under the control of the naturally alkane-inducible AlkR regulator protein. Although the detection limit was higher than that of *P. oleovorans*-based *E. coli* reporter strains [37], the *A. baylyi* ADP1 reporter strain was capable of detecting alkanes between 7- and 36-carbons in

length, giving it a significantly enhanced detection profile [43]. *A. baylyi* ADP1 was also demonstrated to be significantly more tolerant to seawater than *E. coli*, which allowed the reporter to function without any interference from salt contamination. Another advantage of this reporter is that *A. baylyi* ADP1 was shown to adhere to the oil-water interface and emulsify the oil into small droplets by forming a single layer of cells around the droplet surface. This effectively presented an increased sample concentration for the reporter to detect, and aided in decreasing response time to 0.5 h. Testing with alternative contaminants such as salicylate and toluene did not show any significant induction of bioluminescent signal [44], suggesting that the *A. baylyi* ADP1 reporter may be more specific than previous *P. oleovorans*-based versions as well. One year later, similar inroads for the detection of longer chain alkanes were made by Kumari et al. [45], who expressed the *luxAB* genes under the control of the *alk* operon from *Alcanivorax borkumensis* SK2 in *E. coli*. However, they ultimately chose to focus on the development of an EGFP (enhanced green fluorescent protein)-based fluorescent reporter strain in place of the *luxAB*-expressing strain that required the addition of decanal to produce a bioluminescent signal.

2.4 Chlorinated Aliphatic Hydrocarbons

Chlorinated aliphatic hydrocarbons have been widely used throughout the chemical industry for decades. Through mishandling and environmental releases over this time frame, they have become widespread in soils and groundwater [46], and because of their widespread deposition and toxic nature, they now pose a significant risk to both human and environmental health [47, 48]. For these reasons, there is an increasing interest in the development of bioreporters that are capable of determining the location and bioavailability of these toxic compounds in order to direct remediation efforts for their disposal.

Classically, bioreporter-based chlorinated aliphatic hydrocarbon detection mechanisms have been established around the *tod* operon. This operon consists of three genes responsible for oxidation of toluene to cis-toluene dihydrodiol under the control of a regulatable promoter that is upregulated in response to increasing toluene concentrations [16]. Applegate et al. [15] were able to leverage this action by replacing the downstream *tod* genes with a complete *luxCDABE* operon to develop a *P. putida* strain (TVA8) capable of responding to challenges with either toluene or the chlorinated aliphatic hydrocarbon trichloroethylene (TCE) by production of a bioluminescent signal [49]. Using this reporter, it was possible to detect TCE at a lower detection limit between 1 and 5 μM and an upper limit of 230 μM, although the results of the analysis could be easily skewed in the presence of contaminating toluene. Despite this detriment, the *P. putida* TVA8 bioreporter was successfully deployed under environmental conditions, where it was able to detect TCE and 1,1,1 trichloroethane (TCA) in contaminated groundwater samples as confirmed by analytical analysis [20], further demonstrating its utility.

More recently, a second *P. putida* operon has been discovered that can be used as an alternative to the traditional *tod*-based approach. This operon, the *sep* operon, consists of three efflux pump-encoding genes that are regulated in response to a variety of common chemical solvents [50]. When the *luxCDABE* operon was cloned in place of the upstream *sepA* gene, the result was a strain that modulated bioluminescent activity in response to TCE availability. This strain was still susceptible to interference by the same contaminant chemicals as the TVA8 strain [15], however, it did present investigators with another tool for the realtime detection and monitoring of a wide range of halogenated solvents and chlorinated aliphatic hydrocarbons.

Since the time the *tod* and *sep*-based reporter systems were first developed, a more specific reporter has emerged that can sense and respond to the presence of the chlorinated aliphatic hydrocarbon dichloromethane (DCM). The selectivity of this reporter is due to its utilization of the *dcm* operon from *Methylobacterium extorquens* DM4, which is able to grow on DCM as a sole carbon source. The *dcm* operon consists of the genes *dcmAR*, with the *dcmA* gene upregulated in the presence of DCM and the *dcmR* gene encoding a contrasting negative regulatory element. By cloning the *luxCDABE* genes under control of the *dcm* promoter, it was possible to elicit a bioluminescent response from aerosolized DCM at a range between 12 μM and 1.2 mM. Induction of the bioluminescent signal could be observed at 1 h posttreatment at the 1.2-mM level, but increased to 2.3 h at the 12-μM level. In the liquid phase, the reporter could detect DCM between a range of 1.2 μM–12 mM, and the induction time was relatively decreased compared to aerosolized samples, requiring only 0.5 h at the 12-mM concentration. Regardless of the medium used (aerosol or liquid) there was a correlation between bioluminescent output and DCM concentration at an R^2 value of 0.99 [51]. In contrast to the nonspecific reaction of *tod*- and *sep*-based systems, this level of specificity and dose–response kinetics highlights what can be achieved by modulating the selectivity of the upstream regulatory element that is used for bioreporter generation.

2.5 Biphenyl and Polychlorinated Biphenyls

PCBs represent some of the most widely distributed and persistent environmental contaminants due to their resistance to physical, chemical, and biological degradation. It is because of this exceptional stability that PCBs found extensive use as dielectric and coolant fluids in transformers, capacitors, and electric motors. Evidence of their consequent discharge can be found in nearly all environmental ecosystems, including water, sediments, soils, and air, and their tendency to bioaccumulate in living organisms magnifies their presence throughout the food chain as well. Microbiologically, there are a handful of known bacterial species that can utilize biphenyl as a sole source of carbon and energy, predominantly via oxidative degradation mediated by the biphenyl gene cluster (*bph*) [52]. Layton et al. [53]

were the first to exploit the *bph* pathway for PCB bioluminescent biosensing by linking the *bph* R1 regulatory region to a plasmid-localized *luxCDABE* gene cassette that was inserted into *Ralstonia eutropha* to create the bioreporter ENV307(pUTK60). Validation was performed against biphenyl, 2-, 3-, and 4-chlorobiphenyl, and an Aroclor PCB mixture. However, due to the poor aqueous solubility of PCBs, a surfactant was added to the test samples to promote increased bioavailability. Minimum detection limits ranged from 0.80 μM for 4-chlorobiphenyl to 4.6 μM for Aroclor in a 96-well microtiter plate assay over a 6-h incubation period. The need to add surfactants to PCB samples then drove this group to create an improved toxicity-based bioassay because, in the standard Microtox test, the toxicity of the surfactants toward the *A. fischeri* reporters interferes with the toxicity profile of the PCB compounds [54]. To circumvent this limitation, Layton et al. [55] developed their toxicity assay using indigenous wastewater microorganisms displaying surfactant resistance (*Stenotrophomonas* sp. and *Alcaligenes eutrophus*) that were engineered to bioluminesce constitutively via plasmid insertion of a *luxCDABE* gene cassette. Results showed these two strains to be 400 times more resistant than *A. fischeri* to the commonly used surfactant polyoxyethylene 10 lauryl ether, signifying their potential practicality in PCB and other compound toxicity bioassays that require the addition of surfactants.

Pseudomonas sp. DJ-12 expresses a *meta*-cleavage dioxygenase via the *pcbABCD* operon that enables degradation of select biphenyl compounds. Park et al. [56] created plasmid-based gene fusions between the *pcbC* promoter and *luxCDABE* and *luc* to create two bioluminescent bioreporters in *E. coli* host cells. Bioassays performed in 96-well microtiter plates over 30-min exposure periods indicated responsiveness to biphenyl compounds between the 0.1 and 1-mM exposure concentrations analyzed. Testing at lower concentrations to establish true detection limits still needs to be performed, as well as compound specificity studies, but these bioreporters demonstrate a potential addition to the inventory of biphenyl-responsive bioreporters.

In bacteria and higher organisms, PCBs are biotransformed by cytochrome P-450 monooxygenases and metabolized to hydroxylated PCBs (OH-PCBs). Certain bacteria are able to use hydroxybiphenyls as sole carbon and energy sources via mediation of the *hbp* gene cluster under regulatory control of the *hbpR* gene [57]. Recognizing this, Turner et al. [58] linked the *hbpR* gene from *Pseudomonas azelaica* to the *luxAB* genes on a plasmid-based (pHYBP109) system that was inserted into *E. coli* to create a bioluminescent reporter for OH-PCBs. The bioreporter was tested against 27 OH-PCBs with dose-dependent responses successfully obtained with limits of detection in the range from 10^{-5} to 10^{-9} M in 4-h incubation assays followed by the addition of the n-decanal substrate. The bioreporter was also used by Tecon et al. [14] in a *luxAB*-based multibioreporter assay to monitor for oil spill constituents in aquatic ecosystems, subsequently allowing for the simultaneous detection of biphenyls, short-chain linear alkanes, and monoaromatic and polyaromatic compounds within a 3-h assay. Validation of the bioreporter was also applied diagnostically in human serum samples spiked with

an individual OH-PCB (2-hydroxy-3′,4′-dichlorobiphenyl) as well as a mixture of 10 OH-PCBs, with demonstrated detection limits as low as 5×10^{-8} M within a 4-h bioassay time frame [58].

With a goal of expanding the bioreporter's chemical detection portfolio, Tropel et al. [59] then subjected this *hbpR* promoter/operator region to site-directed mutagenesis to modify its recognition specificity. Using this approach, they successfully created an *hbpR-luxAB P. azelaica* bioreporter that was capable of responding to both *m*-xylene and 2-hydroxybiphenyl. This ability to combine different regulatory pathways within a single bioreporter to enable biosensing across different chemical classes facilitates simplified multitargeted bioassays for expanded environmental monitoring.

2.6 Phenol and Derivatives

Phenols and their derivatives serve as some of the most common environmental pollutants in soil, water, and air. Deposition occurs through both natural events (i.e., decomposition of organic material, forest fires, and atmospheric degradation of benzene) and industrial activities where it is produced in massively high volumes (approximately 7 billion kg per year) as an important precursor component of plastics, epoxies, explosives, detergents, herbicides, and pharmaceutical drugs. Under such large-scale manufacturing demands, environmental impacts (especially in relation to wastewater discharges) are well recognized, with 12 phenolic compounds registered on the US Environmental Protection Agency's list of priority pollutants [phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,4-dimethylphenol, 2,4-dinitrophenol, 4-nitrophenol, 2-nitrophenol, pentachlorophenol, 2,3,4,6-tetrachlorophenol, 2,4,6-trichlorophenol, 4-chloro-3-methylphenol (synonym 4-chlor-m-cresol), and 2-methyl-4,6-dinitrophenol (synonym 4,6-dinitro-o-cresol)]. Accordingly, microorganisms have evolved to utilize these phenols, which provides an inroad for the development of bioreporter assays based on their genetic pathways to detect and monitor these phenolic compounds [60]. Over two decades ago, Shingler et al. [61] isolated a *Pseudomonas* strain, CF600, capable of using specific phenols and derivatives as sole sources of carbon. Elucidation of its genetic pathway for doing so described the now well-understood *dmp* operon [62] that later became the platform for the *luxCDABE*-based bioluminescent bioreporter *P. fluorescens* OS8(pDNdmpRlux) [63]. Reporter OS8(pDNdmpRlux) demonstrated a response portfolio to a variety of phenols, including 2-, 3-, and 4-methylphenol (synonyms *o*-, *m*-, and *p*-cresol), 2,3-, 2,4-, 3,4-, and 2,6-dimethylphenol, resorcinol, and 5-methylresorcinol, with maximum detection limits achieved under 2-methylphenol (0.30 μM) and phenol (0.87 μM) exposures of a 4-h duration. As is customary with these types of bioreporters, specific phenols cannot be individually identified and the bioluminescent signal rather represents the total phenolic content in its bioavailable form. When applied to natural, mixed contaminant groundwater and semicoke dump leachates containing primarily

phenol and methylated phenols, the bioreporter successfully bioindicated phenol bioavailability in nine of the ten samples analyzed, with this single negative sample hypothesized to contain phenolic constituents in a non bioavailable form [63]. Building upon these results, Wise and Kuske [64] devised a second generation bioreporter using mutated versions of the regulatory DmpR region of the *dmp* operon to increase the range of phenolics detected. This new reporter construct extended the range of detectable compounds to include 2-chlorophenol, 2, 4-dichlorophenol, 4-chloro-3-methylphenol, and 2- and 4-nitrophenol. Similarly, Gupta et al. [65] performed a more defined DmpR mutation approach and linked gene expression to firefly luciferase within an *E. coli* host cell to create a bioluminescent bioreporter (pRLuc42R) capable of detecting phenol at a lower limit of 0.50 μM within a 3-h assay time frame, however, its ability to detect phenolic compounds in realworld samples has yet to be tested.

Microbes belonging to the genus *Acinetobacter* also utilize phenol as a sole carbon source, and have thus also been transformed into bioluminescent bioreporters. Their genetic architecture consists of a *mop* operon wherein the MopR regulator activates phenol hydroxylase expression upon binding with phenolic compounds such as phenol, 3-chlorophenol, and 2- and 3-methylphenol [66]. Abd-El-Haleem et al. [67] developed the *Acinetobacter* bioreporter DF4-8 via linkage of this regulatory activity to the *luxCDABE* gene cassette to create a bioluminescent bioreporter capable of detecting phenol at a limit of detection of 0.03 mM within an approximate 4-h assay. When exposed to slurries of aged soils obtained from a phenol-contaminated industrial site, the bioreporter elicited a bioluminescent signal within approximately 6 h. This group then further developed a *luxCDABE*-based constitutively bioluminescent *Acinetobacter* bioreporter (DF4/PUTK2) for chemical toxicity assessment and showed its decreasing levels of bioluminescence in response to several phenolic compounds ranging from 50 to 500 ppm ($EC_{50} = 333$ ppm) [68]. They also immobilized the bioreporters in calcium alginate and demonstrated an 8-week storage capacity at 4 °C in a 96-well microtiter plate format, suggesting application toward a prepackaged, off-the-shelf sensor platform. A larger panel of bioreporters for monitoring phenol-related toxicity was developed by Wiles et al. [69] using four wastewater *Pseudomonas* isolates engineered to carry a chromosomally integrated *luxCDABE* cassette. When exposed to natural wastewater effluent samples in a 96-well microtiter plate format under a 5-min incubation period, this panel effectively bioindicated phenolic concentration shifts in concentrations ranging from approximately 10 to 800 ppm ($EC_{50} = 454$–757 ppm). In both this study and the previously described *Acinetobacter* reporter study, toxicity profiles were compared against the standard Microtox assay, where *A. fischeri* is used as the sensor microorganism [70–72], and in both cases Microtox performed less reliably. The advantage of using indigenous Pseudomonads or *Acinetobacter* strains in these studies is their natural robustness to the wastewater environment undergoing testing, whereas *A. fischeri*, being native to the marine environment, is less ecologically adapted and oftentimes responds less efficiently or requires additional sample preparation steps to adjust its performance efficiency [73].

3 Evaluation of Organic Toxicant-Induced Health Risks Using Eukaryotic Cell-Based Bioluminescent Assays

In addition to bacteria, eukaryotic cells, including the lower eukaryotic organism *Saccharomyces cerevisiae*, and cultured mammalian cell lines have been increasingly employed to serve as hosts for bioluminescent reporter assays against organic toxicants. However, unlike in bacterial bioassays where catabolic pathways for the biodegradation of target substances are exploited for reporter development, eukaryotic cells generally are not able to utilize such toxic organic compounds as carbon sources or lack exhaustively characterized catabolic pathways altogether. These deficiencies have been overcome as research on cell biology and toxicology have revealed that many essential receptor-mediated signaling transduction pathways for normal cellular functions can be disrupted by the binding of exogenous organic toxicants to endogenous receptors [74], which thus allows reporter development using similar techniques. Under these strategies, the characterization of key receptors and transcriptional response elements in these pathways forms the foundation for eukaryotic-cell–based bioluminescent bioreporters, which are commonly created by fusing bioluminescent reporter genes to the response element of a target pathway so that bioluminescent expression is transcriptionally modulated through chemical-receptor binding. This design requires coexpression of cognate receptors that are either endogenously present in some cell lines or, in the case of nonexpressing cell lines and *S. cerevisiae*, can be co-introduced with the reporter construct. This means that, because reporter gene expression is integrated into endogenous toxicity pathways, eukaryotic whole-cell bioreporters essentially measure chemical-triggered biological effects, making them ideal tools for providing pathway-specific risk assessment information. This is particularly important during environmental contaminant evaluation where multiple pollutants with a range of toxicological effects often coexist throughout a contaminated site. Because it is common for structurally different chemicals to exert similar toxic effects or for the same compound to induce multiple pathways (i.e., some PCBs have been shown to have both dioxin-like and estrogenic activity [75, 76]), eukaryotic cell-based bioassays can therefore serve as valuable tools for the estimation of potential harmful effects that may not be detectable using traditional analytical means.

Currently, the major use of these eukaryotic cell-based reporters has been for the detection of organic contaminants responsible for dioxin-like (Table 2) or endocrine-disrupting activities (Table 3). This section summarizes a variety of bioluminescent-based bioassays that have been developed for the detection of these two activities. Due to their extensive use in the literature, it is impractical and unnecessary to list all of the applications, however, a focus is drawn on highlighting the predominant application areas with recent examples to provide an overview of the usefulness and limitations of these unique reporter systems.

Table 2 Eukaryotic cell-based bioluminescent bioreporters for dioxin-like chemicals and recent environmental applications

Host cell (organism)	Reporter construct (reporter name)	Detection limit (TCDD)	EC_{50} (TCDD)	References	Recent environmental application
S. cerevisiae	hAhR and hARNT, DRE(×5)-*luc*	1 nM	4.8 nM	[91]	Sewage sludge [135] Sediment [91]
HepG2 (human)	DRE(×4)-*luc*	1 pM	0.35 nM	[82]	Solid municipal waste [136]
H4IIE (rat)	DRE(×4)-*luc* (H4IIE.Luc)	0.5 fM	10 pM	[83]	River water [137] Sediment [137]
Hepa1 (mouse)	DRE(×4)-*luc* (H1L1.1c2)	0.1–1 pM	30 pM	[80]	Storm water [138] Seawater [92]
RTH-149 (rainbow trout)	DRE(×4)-*luc* (RTL 2.0)	4 pM	64 pM	[139]	Seawater [140]
RHEK-1 (human)	DRE(×4)-*luc* (HKY1.7)	10 pM	200 pM	[141]	N/A[a]
Hepa1 (mouse)	DRE(×20)-*luc* (H1L7.5c3)	0.01 pM	10–16 pM	[142]	N/A

[a] N/A not available

3.1 Bioassays for Dioxin and Dioxin-Like Compounds

In their most basic form, dioxins are any compound containing a heterocyclic 6-membered ring consisting of 2 oxygen atoms and 4 alternative atoms. Practically, dioxins are persistent pollutants that can bioaccumulate over time, leading to increased health risks for organisms of higher trophic levels, with the classic example being 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is the most toxic of the known dioxins, and is thus used as a representative model, with the relative toxicity of other chemicals expressed in toxic equivalency factors [77]. Collectively, chemicals that inflict toxic effects similar to TCDD are classified as dioxin-like, and can lead to hepatotoxicity, embryotoxicity, teratogenicity, immunotoxicity, dermal toxicity, carcinogenesis, or lethality [78, 79]. Dioxin-like activities have been found in various groups of organic compounds, including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), some PCBs, and PAHs.

The mechanism of action for dioxin-regulated gene expression begins when the chemical binds to the aryl hydrocarbon receptor (AhR) in the cytosol. This chemical–AhR complex then translocates into the nucleus and forms a dimer with an AhR nuclear translocator (ARNT) protein. The dioxin:AhR:ARNT complex then binds to specific DNA sequences called dioxin-responsive elements (DREs), which results in the activation of the adjacent responsive gene(s) [80]. Because the resulting increase in gene expression is directly proportional to the toxicity of the

Table 3 Eukaryotic cell-based bioluminescent bioreporters for endocrine-disrupting chemicals and recent environmental applications

Host cell (organism)	Reporter construct (reporter name)	Detection limit	EC_{50}	References	Recent environmental application
Estrogenic and antiestrogenic					
S. cerevisiae	hERα, ERE(×2)-*luxCDABE* (BLYES)	45 pM E2	0.24 nM E2	[118]	Freshwater [143] Drinking water [143, 144]
S. cerevisiae	hERα, ERE(×2)-*luc* (BMAEREluc/ERα)	30 pM E2	0.5 nM E2	[116]	Wastewater [145]
S. cerevisiae	hERβ, ERE-*luc* (BMAEREluc/ERβ)	0.1 nM E2	0.5 nM E2	[116]	N/A[a]
MCF-7 (human)	ERE-*luc* (MVLN)	1 pM	20 pM	[98, 99]	River water [137] Sediment [137] Wastewater [146]
MCF-7 (human)	ERE-*luc* (MELN)	1 pM E2	5 pM E2	[102]	Wastewater [125, 147] Freshwater [148, 149] Sediment [148, 149]
T-47D (human)	ERE(×3)-*luc* (T-47D ER-CALUX)	0.5 pM E2	6 pM E2	[104]	Wastewater [150, 151] Freshwater [151, 152] Sediment [151]
T-47D (human)	ERE(×3)-*luc* (T-47D-KBluc)	1 pM E2	10 pM E2	[105]	Wastewater [126, 153, 154] Freshwater [153]
HeLa (human)	hERα, ERE-*luc* (HELNα)	1 pM E2	5 pM E2	[102]	Drinking water [155]
HeLa (human)	hERβ, ERE-*luc* (HELNβ)	1 pM E2	10 pM E2	[102]	N/A
U2-OS (human)	hERα, ERE(×3)-*luc* (ERα-CALUX)	0.8 pM E2	20 pM E2	[113]	Indoor dust [156] Drinking water, wastewater, and freshwater [157]
Androgenic and anti-androgenic					
S. cerevisiae	hAR, ARE(×4)-*luxCDABE* (BLYAS)	2.5 nM DHT	9.7 nM DHT	[119]	N/A

(continued)

Table 3 (continued)

Host cell (organism)	Reporter construct (reporter name)	Detection limit	EC_{50}	References	Recent environmental application
S. cerevisiae	hAR, ARE(×2)-*luc* (BMAAREluc/AR)	50 pM testosterone	10 nM testosterone	[116, 117]	Sediment [137]
		0.5 nM DHT	5.5 M DHT		
T-47D (human)	ARE(×2)-*luc* (AR-LUX)	46 pM methyltrieno-lone	86 pM methyltrieno-lone	[107]	Freshwater [158]
MDA-MB-453 (human)	MMTV-*luc* (final reporter known as MDA-kb20	0.1 nM DHT		[108, 126]	Wastewater [126, 159]
O2-US (human)	hAR, HRE (hormone response element)-*luc*	3.6 pM DHT	0.13 nM DHT	[113]	Drinking water, wastewater, and freshwater [157]
Glucocorticoid receptor (GR) agonist and antagonist					
MDA-MB-453 (human)	MMTV-*luc* (final reporter known as MDA-kb2)	10 nM dexamethasone	N/A	[108]	Wastewater [159]
O2-US	hGR, HRE(×3)-*luc* (final reporter known as GR-CALUX)	0.2 pM dexamethasone	0.37 nM dexamethasone	[113]	Wastewater [160]
Thyroid receptor (TR) agonist and antagonist					
PC12 r(rat)	Avian TRα1, TRE(× 4)-*luc* (PC-DR-LUC)	30 pM 3,3',5-triiodo-$_L$-thyronine (T3)	0.18 nM T3	[161]	Wastewater, drinking water, and surface water [162]
Progesterone receptor (PR) agonist and antagonist					
O2-US	hPR, HRE(×3)-*luc* (PR-CALUX)	1.3 pM Org2058	0.09 nM Org2058	[113]	Indoor dust [156]
					Drinking water, wastewater, and freshwater [157]

[a] N/A not available

binding chemical [81], this process provides an excellent platform for the development of regulatable bioluminescent bioreporter-based dioxin detection strategies.

The first dioxin-responsive bioluminescent reporter to be developed was used by Postlind et al. [82] to track expression of the *CYP1A1* and *CYP1A2* genes of the human cytochrome P450 gene family. This was accomplished by cloning the 5′ flanking region from either the *CYP1A1* or *CYP1A2* genes upstream of a *luc* gene in a human expression vector. These vectors were then introduced to a human hepatoma (HepG2) cell line and challenged with TCDD and other compounds. Under transient transfection conditions where cells were exposed to TCDD for 24 h the day after plasmid introduction, reporters expressing the *CYP1A2* 5′ flanking sequences displayed a detection minimum of 0.01 nM TCDD, whereas those expressing *CYP1A1* 5′ sequences were detectable down to a concentration of 0.001 nM. Each was capable of responding to TCDD treatment in a dose–response fashion, however, although the signal maximum for the *CYP1A2*-based reporter was 10-fold over control upon treatment with 10-nM TCDD, the *CYP1A1*-based reporter reached its signal maximum at 65-fold over control upon 100-nM treatment. This gave the *CYP1A1*-based reporter both a larger range of detection, as well as a greater signal intensity throughout that range. Commensurate with its lackluster performance compared to *CYP1A1*, the *CYP1A2*-based reporter construct was not able to function at all under stable expression conditions. Whereas the CYP1A1-based reporter was capable of stable expression and could detect 10-nM TCDD at 0.5 h postexposure, with increasing reporter activity positively correlating with exposure time up to 24 h. The EC_{50} for TCDD detection by this reporter was determined to be 0.35 nM, making it the first functionally useful dioxin-detecting human cell line.

Building upon this expression strategy, Garrison et al. [80] used a construct consisting of a mouse mammary tumor virus promoter mediated *luc* gene under the control of a 484 base pair 5′ upstream mouse *Cyp1a1* gene sequence that contained 4 DREs to generate a range of bioluminescent dioxin-responsive bioreporter cell lines. When treated with 1 nM TCDD for 24 h the day after plasmid introduction, all of these lines were capable of responding to TCDD challenge with a corresponding bioluminescent output. This demonstrated that the mouse DREs could be successfully recognized by the dioxin:AhR:ARNT complex across a wide range of species, and therefore that the assay could be performed in specific cell types to determine species-specific bioavailability of dioxins and dioxin-like compounds. Bolstered by these findings, a second set of stably transfected dioxin-responsive bioluminescent bioreporter cell lines were developed. Of these cell lines, the mouse H1L1.1c2 line (Hepa1-derived) was chosen as a model for characterization because it had the greatest level of induction and was found to respond reliably to TCDD treatment in a dose–response fashion (although this dose–response relationship was also reported for the human HepG2-derived HG2L1.1c3 cell line, no data were presented to support this claim at the time). The model H1L1.1c2 reporter cell line displayed a minimal detection limit between 0.1 and 1.0 pM of TCDD, with a maximal induction of 80-fold over

control at 1.0 nM, providing an ED_{50} of 0.02 nM. This is less than the previous value of 0.35 nM established by Postlind et al. [82] with their human HepG2 cell line, and represented a lower maximal detection limit (1.0 vs. 100 nM) as well. Also unlike the Postlind study, Garrison et al. [80] were not able to detect a bioluminescent signal until 2 h posttreatment, and reached a maximum induction at 4 h posttreatment following application of 1.0-nM TCDD. However, despite being mostly in line with the Postlind study, the Garrison study remains notable for its demonstration of bio-reporter function across a wide variety of cell lines, and for demonstrating that the inducing chemicals did not act as competitive substrates for the luciferase enzyme, providing a significant advantage for the bioluminescent assay over older, more traditional chemical-based assays.

Following up these successful demonstrations against specific chemicals in laboratory settings, Murk et al. [83] transported the system to a rat hepatoma H4IIE cell line and rebranded its use as a chemical-activated luciferase expression (CALUX) assay. Using this new cell line they were able to demonstrate TCDD detection down to 0.5 fM, with a maximum detection limit between 100 pM and 1.0 nM, and an EC_{50} of 10 pM. Similar to the earlier experiments [80, 82], they were also able to demonstrate a dose-response relationship between TCDD and bioluminescent signal, and did not discover any significant substrate inhibition.

What solidified the CALUX assay as the predominant method for dioxin-like compound detection, however, was the successful demonstration of its use with environmental samples and its ability to mimic the results of existing, more complex and more costly in vivo assay results [83]. Murk and colleagues demonstrated that the *luc*-expressing H4IIE reporter cells could be exposed to extracted sediment and water samples to determine toxic equivalency factors rapidly and inexpensively. Although it was ultimately determined that unpurified sediment samples could become toxic to the H4IIE-*luc* cells, it is primarily highlighted that purification was not required for water samples, which significantly improved the speed at which they could be assayed. Perhaps more important, however, was the validation of the CALUX assay against the in vitro zebrafish early life stage assay. Although the presence of heavy metals led to a poor correlation between the two assays when performed directly in unprocessed environmental water samples, this correlation significantly improved following sample extraction. When compared side by side under laboratory controlled conditions, the EC_{50} as determined by the zebrafish early life stage assay was found to be 21 pM, whereas the EC_{50} of the CALUX assay was 10 pM. Moreover, the working range for the CALUX assay was improved compared to the zebrafish early life stage assay, and reduced both the time and cost involved in its performance.

Through its low cost, lack of substrate inhibition, ability to be adapted for high-throughput, and ability to function in disparate cellular backgrounds, the CALUX assay has become the predominant method for assaying dioxin and dioxin-like chemicals using mammalian cells. Since its early demonstrations as a tool for laboratory-based chemical toxicity testing and environmental pollutant screening, it has been used for a wide variety of applications including veterinary [84], food product testing [85–88], and human clinical sample analysis [89, 90]. And as the

applications for the CALUX assay have expanded, so have the organisms that have been employed for its use. Although still predominantly performed in mammalian cell lines, recently the assay has been reconstituted in yeast by co-expressing the genes for AhR and ARNT with a DRE-mediated *luc* luciferase. This switch away from human cells and into the microbial eukaryote *S. cerevisiae* was done with the hope that it would provide a more robust and simplified expression system that could improve deployability and reduce costs. It was found that the yeast-based system could respond to TCDD treatment in a dose–response fashion, with a minimum detection time of 3.5 h and an EC_{50} value similar to that of the early human cellular reporters at 4.8 nM. Inasmuch as this assay was validated against the classical H4IIE-*luc* cell line-based CALUX assay and found to be in good agreement [91], it has since been used as a first stage in vivo screen for dioxin-like chemical load detection in composted sewage sludge [91], proving its worth as an alternative means of detection when human cell lines cannot be used.

As a final note, with the use of CALUX assays continuing to proliferate, it is important to recognize that the results of the assay can vary from lab to lab, and even from run to run [92]. In a validation study, Besselink et al. [93] found 14.6 and 26.1 % intralaboratory assay reliability levels for pure compounds and whole matrix, respectively, and 6.5 and 27.9 % interlaboratory assay reliability levels for pure compounds and whole matrix, respectively. Therefore, in light of these discrepancies, it is important to review the sample cleanup methods, the effects of the solvents used during extraction and testing, any known interaction with synergistic or antagonistic compounds used, the cell line utilized, and the analysis methods employed before comparing results between multiple sources in order to determine the validity of the comparison [92]. Despite these caveats, however, the CALUX assay remains the predominant method for bioluminescent screening of compounds eliciting dioxin-like activities.

3.2 Bioassays for Hormonally Active Chemicals

The vertebrate endocrine system consists of a group of signaling molecules collectively called hormones, which act through binding to their corresponding nuclear receptors in order to transcriptionally modulate expression of genes involved in different stages of an animal lifecycle including development, growth, and reproduction. Due to the critical roles of hormones, any interference with the endocrine system may lead to various adverse health effects in humans and wildlife [74], and a variety of organic compounds has been discovered that are considered as endocrine disruptors because of their ability to mimic or repress the function of these natural hormones in vivo [94, 95]. The presence of these chemicals in plastics, pesticides, herbicides, household products, cosmetics, and pharmaceuticals, and their wide use and discharge into the environment through anthropogenic activity and industrial waste has made the detection of endocrine disruptive activity an increasingly large concern.

The major mechanism of action for endocrine disrupting chemicals (EDCs) is to modulate the transcriptional activity of hormone receptors, which includes (but is not limited to) estrogen receptors (ER—with two isoforms ERα and ERβ), androgen receptors (AR), glucocorticoid receptors (GR), progesterone receptors (PR), and thyroid receptors (TR). Because of this mechanism of action, bioluminescent bioreporters for EDCs are constructed similar to the dioxin bioreporters by conditionally expressing a reporter gene (either *luc* or *luxCDABE*) under the control of the corresponding response element. Therefore, detection of organic compounds using these bioassays is activity—rather than structure-oriented. Cell-based reporter gene assays for EDCs including bioluminescent bioassays are routinely reviewed in the literature (for recent examples see [96, 97]) and are summarized here in Table 3.

Depending on the source of the target receptors, these bioreporters can be divided into two types, those that exploit endogenous receptors and those that require manual receptor cointroduction. For those that exploit endogenous receptors, the ER-positive human breast cancer cell lines MCF-7 and T-47D are the model platforms for bioassays of estrogenic and antiestrogenic activities. The first stable bioluminescent bioreporter for ER agonists and antagonists in mammalian cells was the MCF-7-derived MVLN reporter cell line developed by Pons et al. [98] and subsequently shown to be responsive to both estrogenic and antiestrogenic substances [99]. In these reporter cells, *luc* gene expression was placed under the control of the estrogen response element (ERE) derived from the 5′ flanking region of the *Xenopus* Vitellogenin A2 gene. Due to the limited techniques available at that time, the original tests with these cells were performed in a now rarely used 6-well plate format. Nevertheless, under these conditions treatment with the natural estrogen 17β-estradiol (E2) exhibited an EC_{50} of ~20 pM after a 24-h exposure period. However, as technology has improved, this assay has been modernized, and is now commonly carried out in a more standard 96-well plate format and with a 48-h exposure [100, 101]. Concurrent with this modernization has been the introduction of additional bioreporter cell lines that function in a similar manner. For instance, building upon the work of Pons et al. [98], Balaguer et al. [102] later developed a similar MCF-7-derived MELN reporter cell line, in which *luc* expression was linked to an ERE and a β-Globin promoter. After 16 h of incubation, the MELN cell line was able to detect E2 concentrations as low as ~1 pM with an EC_{50} value of ~5 pM when performed in a 24-well plate. Other chemicals including a nonylphenol mixture, 4n-nonylphenol, 2,4′-dichlorodiphenyldichloroethylene (2,4′-DDE), and 4,4′-DDE (both of which are DDT breakdown products) also tested positive for estrogenic activity under the same assay conditions. The reproducibility and lab-to-lab variation of the MELN assay against a battery of compounds with known estrogenic or antiestrogenic activity was recently tested in a higher throughput 96-well plate format [103], and this study revealed that, although the individual laboratories maintained mean intralaboratory coefficients of variations of either 32.1 or 56.8 % for their EC_{50} values, both labs produced similar rankings of estrogenic or antiestrogenic potency of most of the chemicals, highlighting the utility of this assay for comparative applications.

Moving away from the MCF-7 cell line, Legler et al. [104] developed a T-47D cell-based ER-CALUX reporter cell line for detection of ER agonists and antagonists. Using a minimal TATA box promoter and three tandem repeats of ERE, this cell line (T-47D ER-CALUX) displayed very low background bioluminescence in solvent controls and a maximum induction of approximately 100- and 76-fold compared to unexposed background following a 24-h exposure to 30 pM E2 in 24- and 96-well plate formats, respectively. This bioassay was also capable of detecting E2 down to ~0.5 pM and is the most sensitive among reported estrogen-specific assays. A similar T-47D-KBluc reporter developed by Wilson et al. [105] has a comparable EC_{50} value for E2 (10 pM in KBluc vs. 6 pM in EREtata-luc) but possesses a larger dynamic detection range from 1 pM to 100 nM. However, despite these similarities, it is worth noting that controversial results were obtained in a follow-up study comparing the in vitro T-47D ER-CALUX assay with an in vivo transgenic zebrafish assay expressing the same reporter construct [106]. Despite the synthetic estrogen 17α-ethynylestradiol (EE2) testing 100 times more potent than E2 in the transgenic zebrafish assay, it showed equal estrogen agonistic activity compared to E2 in the cell-based in vitro assay. One possible explanation for these differences is that the binding affinities of test compounds may be different when interacting with ERs that have originated from different species, which would explain the poor performance of human cell-based assays to predict toxicokinetics in zebrafish models.

Under similar development strategies, bioluminescent bioreporters screening for AR agonists and antagonists have been generated as well. These reporters take advantage of AR-positive cell lines, such as T-47D and the human breast cancer cell line MDA-MB-453 in order to supply the receptors needed for successful activation of their chosen bioluminescent expression systems. Blankvoort et al. [107] were the first to develop a stable T-47D AR-LUX cell line for androgenic and antiandrogenic effects by using a rat probasin promoter-derived ARE-mediated *luc* reporter construct. This reporter cell line was shown to be capable of detecting methyltrienolone down to 46 pM after 24 h of exposure and detecting the environmentally relevant antiandrogenic compounds 4,4′-DDE and (RS)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione (vinclozoline) as well. However, because of the coexpression of other hormone receptors (such as ERα, ERβ, and PR) in the T-47D cellular background, there remains a possibility that nonspecific responses may have been detected. To overcome this issue, Wilson et al. [108] developed a bioassay for chemicals mimicking/blocking androgen and glucocorticoid activities utilizing the MDA-MB-453 cell line, which expressed high levels of AR and GR but showed undetectable or very low levels of alternative receptors [109, 110]. The resulting reporter cell line, named MDA-kb2, expressed the *luc* gene under the regulation of an AR- and GR-responsive mouse mammary tumor virus (MMTV) promoter and, because of the characteristic low-level expression of competing hormone receptors, provided a significant decrease in background activity that led to an increased signal-to-noise detection ratio.

This decrease in alternative receptor expression resulting from the use of the MDA-kb2 cell line helped to reduce cross-talk between different pathways [111],

but was not the only means for accomplishing this goal. With the hope of providing a more receptor-specific bioassay, several cell lines with little to undetectable levels of nontarget receptors have been utilized through the introduction of specific receptors that are not natively expressed. Examples of this approach include the use of human cervical cancer cell line HeLa and osteosarcoma cell line U2-OS as parental cells for reporter development. In addition to the MELN reporter cell line, the HELNα and HELNβ bioreporters were developed by Balaguer et al. [102] by cointroducing ERα and ERβ, respectively. Although the E2-generated bioluminescent response of these cell lines is similar to that observed in MELN assays, it should be noted that TCDD can elicit an antiestrogenic response in HeLa cell-based assays compared to its demonstrated estrogenic activity in MELN assays. This differential behavior highlights an example of the effect of pathway cross-talk (in this case between the AhR- and ER-mediated pathways), which must always be accounted for during data interpretation. To reduce the prevalence of this cross-talk, a panel of CALUX bioassays has subsequently been developed for selective detection of chemicals interacting with ERα, ERβ, AR, GR, and PR using the U2-OS cell line, which demonstrates little or no natural activity of any of these receptors [112–114]. These reporter cell lines were generated by cointroducing a vector that conferred constitutive receptor expression, and a second vector that permitted target receptor-mediated expression of the *luc* gene. Using this approach, the ERα- and AR-CALUX bioassays were also shown to be well correlated with other animal-based assays (R^2 value of 0.46 and 0.87 for AR and ERα assays, respectively), making them useful tools for predicting potential in vivo activities with a reduced chance of cross-talk–based interference [115].

For similar reasons to those listed above, as the techniques for genetic expression continue to improve, there has been an increased interest in using the lower eukaryotic organism *S. cerevisiae* as a platform for hormonally active chemical screening. Due to their lack of human hormone receptor expression, fast and robust growth, and relatively simplified genetic manipulation techniques, yeast-based bioreporters can now be constructed using a stepwise transformation of a recombinant human hormone receptor (e.g., hERα, hERβ, and hAR) of interest and a receptor-responsive reporter gene. Several *luc*-based yeast bioreporters have been developed for the rapid profiling of estrogenic and androgenic potentials, and have demonstrated a lower detection limit for E2 and dihydrotestosterone of 30 and 50 pM, respectively [116, 117]. Despite this reduced sensitivity compared to mammalian cell-line–based assays, the yeast-based assays were capable of reporting relative potency of test chemicals more rapidly, with only a 2.5-h incubation time.

In particular, two yeast bioassays, BLYES [118] and BLYAS [119], which have been developed to detect estrogenic and androgenic activity, respectively, stand out from the other bioreporters mentioned above with respect to their choice of bioluminescent reporter genes. Instead of using the *luc* reporter gene, each of these utilizes a bioluminescent end point resulting from expression of the *luxCDABE* genes. The switch to the *lux* system eliminates the need for exogenous luciferin addition and/or cell lysis and permits autonomous bioluminescent signal

generation and detection. This allows the assay to proceed more rapidly, and with near realtime signaling. Bioluminescent signal detection can occur as early as 1 h after exposure to 2.8 nM E2 in the BLYES assay [118], which allowed the BLYES and BLYAS assays to be used to evaluate the toxicity and potential endocrine-disrupting activities of a battery of 68 chemicals quickly and efficiently in a cost-effective manner [120].

Compared to bioreporters relying on endogenous receptor-mediated signaling, the test responses generated using recombinant yeast bioreporters and mammalian bioreporters with manually introduced receptors are less likely to be subject to nonspecific interactions, which may provide improved mechanistic insight. However, it still remains to be seen if an enhanced prediction of in vivo effects might be achieved using bioassays without exogenous manipulation of the signaling receptors. This creates a potential tradeoff that will need to be evaluated on a case-by-case basis.

3.3 Environmental Applications

The discharge of chemicals through anthropogenic activity and industrial waste in the environment urges a careful assessment of ecologically relevant compounds for their potential toxic effects. One particular phenolic compound of recent emerging importance is bisphenol-A (BPA). BPA is used extensively in the production of polycarbonate plastics and is widely and controversially implicated in causing negative health effects due to its biological action as an endocrine disruptor [121]. Its presence in drinking and wastewaters has become particularly relevant, along with other mid- to long-chain alkylphenols and alkylphenol ethoxylates that have been suggested to exhibit similar properties. For these reasons, a number of bioluminescent reporter systems using both mammalian cell lines and yeast have recently been employed to characterize its endocrine-disrupting activity. Michelini et al. [117] used a yeast-based hAR/ARE-*luc* bioassay to demonstrate the antiandrogenic potency of BPA with a half maximal inhibitory concentration (IC_{50}) of 5 μM against 10 nM testosterone. BPA's estrogenic potential was later supported by a number of different estrogen assays, yielding EC_{50} values of 2.8 and 0.8 μM using the BLYES [118] and T47D ER-CALUX [104] assays, respectively. In addition, several widely used industrial compounds, such as polyfluorinated iodine alkanes, nonylphenol isomers, and phthalates have also been evaluated by the MVLN and H4IIE-*luc* reporter assay for their estrogenic and dioxin-like potentials [101, 122–124]. These uses, although by no means exhaustive, highlight the utility of these eukaryotic cell-based bioluminescent bioassays to offer a high-throughput and relatively inexpensive route for profiling the potential toxicities of the ever-expanding number of chemicals that are routinely being used and released to the environment, providing valuable preliminary data for assessing the deleterious effects of their exposure.

The continuing release of organic compounds through urban and industrial wastewater has also raised concerns regarding the efficiency of its associated treatment processes, as any residual toxic chemical present in the treated effluent is directly discharged into surface water and can thus affect downstream aquatic ecosystems, and in some cases, drinking water supplies. Of particular interest has been the generation of disinfection by-products from oxidative chemical treatment and their potential as endocrine disruptors. To help elucidate this issue, several bioluminescent bioassays have been applied to determine the endocrine-disrupting potencies of industrial wastewater before and after ozonation treatment. For example, Schiliro et al. [125] utilized the MELN assay along with another non-bioluminescent cell-based proliferation assay to measure the E2 equivalents of pre- and postozone treated wastewater from a textile industrial wastewater treatment plant. The MELN bioluminescent bioassay estimated an average of 15.34 (±13.00) pM and 9.29 (±9.10) pM E2 equivalents in pre- and post-ozonation samples, respectively. Although they did note some discrepancy between the measured E2 equivalent values between the MELN assay and the proliferation assay (8.62 (±6.16) pM preozonation and 2.64 (±2.13) pM postozonation), they pointed out that both assays identified a comparable degree of reduction in estrogenic potentials as a result of the ozonation process. Furthermore, with respect to the possible generation of more toxic by-products during ozonation of naphthenic acid, a primary organic constituent of the wastewater produced during the hot water extraction of bitumen from oil sands in surface mining operations, He et al. [126] compared the endocrine-disrupting activity between untreated and ozone-treated oil sands process-affected water. This study demonstrated that antiandrogenic activity was reduced in ozone-treated water compared to untreated water through the use of a MDA-kb2 bioreporter assay. However, unlike in the study of Schiliro et al., and in disagreement with the general idea that ozone disinfection is an effective means to reduce estrogenicity [127], it was reported that the estrogenic potential of the oil sands wastewater was not affected by ozonation when evaluated with the T47D-KBluc bioreporter assay. It is therefore worth noting that wastewater treatment efficiency varies on a case-by-case basis, and that bioluminescent bioassays should therefore be used as a rapid preliminary screening method before applying comprehensive chemical analyses.

Last but not least, to help better understand the potential risks of chemical exposure, eukaryotic cell-based bioluminescent bioassays are increasingly being utilized in combination with chemical analysis to survey ecosystems affected by the discharge of toxic chemicals. Traditionally the pollutant composition would be fully characterized instrumentally, however, bioluminescent bioassays now provide a rapid and cost-effective means of simultaneously assessing potential biological effects as well. In addition, with an increased understanding that additive and possible synergistic effects of complex mixtures could contribute to the overall environmental impact [128], there is an understanding that chemical analysis alone may not generate sufficient information for risk assessment. Similarly, unidentified hormonal activity may be overlooked by instrumental analysis, which only looks for known targets. This was demonstrated by Fenet et al. [129] when they linked

the concentrations of alkylphenols quantified by GC/MS with their contribution toward the total estrogenic activity in environmental samples using the MELN reporter assay. This study recognized varying degrees of correlation between chemically determined concentrations and total estrogenicity on a sample-by-sample basis, demonstrating that, although alkylphenols of GC/MS-determined concentrations could explain a large part of the estrogenic potency in the studied sediment samples, their abundance only provided little to a very low contribution toward the overall observed estrogenic effects in the water samples. These findings have since been repeated, suggesting the presence of other unintended or unknown estrogenic contaminants in studied sites [100]. However, despite their utility to assess biological effects rapidly, it is critical to acknowledge that these bioluminescent assays are not capable of identifying the causative agents, and therefore should not be used as a stand-alone technique for environmental evaluation. A comprehensive assessment of organic pollution requires thorough chemical and toxicological analyses and is often time-consuming. Therefore, the major role of cell-based bioluminescent assays should be to serve as a rapid and economical initial screening tool to prealert samples eliciting positive responses for further investigations and to reduce expense and labor on samples with negative responses.

4 Conclusions

Equipped with a bioluminescent reporter system, living whole-cell bioreporters are capable of sensing the presence of detrimental organic contaminants and internally transforming that input cue into an output signal in the form of light for easy detection. Compared to chemical analysis using costly instrumentations and complicated protocols, bioluminescent bioreporter-based assays are inexpensive, easy to perform, and capable of rapid and high-throughput detection. Bioreporters are often criticized for their compromised specificity, but it is important to note that they are not intended to replace analytical methods for the identification of the exact composition of a contaminated sample. Unlike analytical approaches, which can define structures and measure concentrations, whole-cell bioreporters are designed to survey biological potentials such as the biodegradation and toxicity potentials that are measured by catabolism-based bacterial reporters and toxicology-based eukaryotic reporters, respectively. With the major concerns of environmental monitoring being contamination evaluation and risk assessment, the most suitable application of bioluminescent bioassays is for the rapid prescreening of large numbers of samples to prioritize them for further in-depth examinations in combination with other analyses (including analytical methods) to provide biologically relevant data for comprehensive risk assessments. Realizing that eukaryotic-based bioreporters provide more human- centric biologically relevant information than the bacterial-based bioreporters, there is greater motivation toward their application in establishing toxicokinetic profiles for improved

surveillance and modeling of human/animal health impacts. This includes both the lower eukaryotic yeast and mammalian cell lines that almost exclusively rely upon firefly luciferase as a signaling element, although newer versions of "humanized" bacterial luciferase capable of being expressed under eukaryotic genetic controls without the necessary addition of a light-activating substrate are becoming available for higher throughput, more data intensive, realtime chemical toxicity profiling [130]. As the inventory of bioluminescent bioreporters expands toward more chemical targets with greater specificity, sensitivity, and human relevance, it is clear that the bioreporter's role as an environmental sentinel is here to stay.

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Part III
Applications of Bioluminescence in Agriculture and Bioprocess

Detection of Bacteria with Bioluminescent Reporter Bacteriophage

Jochen Klumpp and Martin J. Loessner

Abstract Bacteriophages are viruses that exclusively infect bacteria. They are ideally suited for the development of highly specific diagnostic assay systems. Bioluminescent reporter bacteriophages are designed and constructed by integration of a luciferase gene in the virus genome. Relying on the host specificity of the phage, the system enables rapid, sensitive, and specific detection of bacterial pathogens. A bioluminescent reporter phage assay is superior to any other molecular detection method, because gene expression and light emission are dependent on an active metabolism of the bacterial cell, and only viable cells will yield a signal. In this chapter we introduce the concept of creating reporter phages, discuss their advantages and disadvantages, and illustrate the advances made in developing such systems for different Gram-negative and Gram-positive pathogens. The application of bioluminescent reporter phages for the detection of foodborne pathogens is emphasized.

Keywords Reporter bacteriophage · Luciferase · Pathogen detection · Foodborne pathogens

Abbreviations

d	Day(s)
g	Gram(s)
h	Hour(s)
L	Liter(s)
min	Minute(s)
ml	Milliliter(s)
mol	Mole(s)
s	Second(s)

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1 Introduction

Bacteriophages are viruses that solely infect bacteria and rely on the host metabolism for reproduction, making them perfect intracellular parasites. They represent the most abundant biological entities on earth and are a major driving force for bacterial evolution [1, 2]. Most bacteriophages belong to the order *Caudovirales*, the tailed bacteriophages, with the three families *Siphoviridae*, *Myoviridae*, and *Podoviridae*. Bacteriophages propagate themselves by adsorption to a host bacteria cell, penetration of the cell wall, and subsequent injection of the phage DNA or RNA. The phage can follow a temperate lifestyle, which is characterized by the integration of the phage genome into the host chromosome and coreplication with the bacterial chromosome. In contrast, a lytic lifestyle results in reprogramming of the host cell by the invading DNA, resulting in production of new virus progeny and subsequence cell lysis. Temperate phages (in the integrated state called "prophage") can excise their genome from the host chromosome under adverse physiological conditions and enter the lytic cycle, thereby eventually killing the host. In both cases, phage DNA is transduced into the host cell during the initial infection, a basic step and prerequisite for development of any reporter bacteriophage.

Bacteriophages have been widely used for therapeutic purposes and for diagnostics of pathogenic bacteria for many years. Phage therapy has been one of the pillars of eastern Europe and Soviet Union healthcare systems but the concept was completely abandoned in the Western world due to the invention of antibiotics [3, 4]. The current crisis caused by antibiotic-resistant bacteria prompted a reconsideration of bacteriophage therapy, and sparked a new area of research using these viruses. In addition to application in medical settings, bacteriophages are becoming increasingly popular for biocontrol of pathogenic bacteria in food. Multiple studies demonstrate the feasibility of the approach in controlling *Salmonella*, *Campylobacter*, *Listeria*, and many other pathogens in a variety of foods, as well as in livestock [5–9]. Also, enzymatic components of the phage, such as endolysins, can be used to control infections and remove unwanted pathogens [10, 11].

Bacteriophages or their components also exhibit great potential for development of diagnostic assays targeted at bacterial pathogens. One especially intriguing method of using phages to detect bacteria are so-called "reporter phages." Here, the phage is genetically modified to carry and transduce a reporter gene, which can be a fluorescent or luminescent marker or any other detectable gene product, which is expressed upon infection of the bacterial host. Such reporter phages have been developed on the basis of firefly luciferase [12], bacterial luciferase [13], beta-galactosidase [14, 15], green fluorescent protein [16, 17], and ice nucleation protein [18]. Monitoring the product or substrate converted by the action of the reporter enzyme enables realtime detection of low numbers of viable bacteria.

Recent large outbreaks of foodborne pathogens, such as the EHEC contamination of sprouts in Germany in 2011 or the occurrence of *Listeria monocytogenes* on cantaloupe melons in the United States in 2012, prompted a quest for simple, specific, and sensitive detection methods for the food industry. Current detection methods for bacterial pathogens from foods still mostly rely on the conventional enrichment and plating methods, which suffer from a number of drawbacks, such as long incubation times (24–48 h, depending on the type of enrichment needed), error-prone procedures, costly reagents, and low specificity. A number of molecular methods for pathogen detection have been developed in recent years, such as PCR or other nucleic acid amplification-based assays, molecular probes, or various immunoassays. Although massively reducing the time-to-result and (in some cases) the detection limit, some assays lack specificity, and cross-reactivity in immunoassays may be problematic. Others, such as PCR-based assays or DNA hybridization technologies offer quick and highly specific results, but are unable to distinguish between live and dead cells and often require cost-intensive equipment and expert staff. Moreover, these procedures are often hampered by the complexity of the sample matrix and uneven distribution of the pathogen in the test sample [19].

Reporter bacteriophages offer a sensitive and efficient alternative, featuring a number of advantages: they are highly specific, robust, and easy to handle, and detect only live cells. Bioluminescent reporter bacteriophages especially require very little processing time until sample testing. Although a suitable substrate and instrument for signal recording is required, the assay is generally inexpensive, and trained staff is not needed to perform the analysis. Furthermore, most foods are unlikely to feature a background activity that would mask or yield false-positive signals in bioluminescence measurements. Last, most bacteriophages can be manufactured with ease in large quantities, rendering the production of the detection agent very cost-efficient. Attempts to replace the luminometer machine by inexpensive Polaroid films or off-the-shelf digital cameras showed great potential in further simplifying the assay procedures [20, 21].

In this chapter, we provide a summary of the current knowledge on bioluminescent (and other) reporter bacteriophages, and their application for diagnostics of bacteria from food and other environments.

2 Bioluminescence as Reporter System

Bioluminescence is mostly mediated by the luciferase enzyme, and the genes encoding the components of this system can be found in different organisms. Most widely used are the *luxA* and *luxB* genes from *Vibrio fischeri* and *Vibrio harveyi* (Lux), as well as the firefly luciferase (Luc) from *Photinus pyralis*. Both catalyze a different light-emitting reaction (see below). The *luxA* and *luxB* genes encode the two subunits for the heterodimeric bacterial luciferase enzyme. In some organisms, the enzyme works best if expressed from a fused *luxAB* gene, instead of individual genes [22–24]. The other components encoded on the *lux* operon (*luxCDE*) are necessary for the conversion of medium-chain fatty acid to the aldehyde substrate to be oxidized by the luciferase. Because the substrate can easily be supplied exogenously for reporter phage assays, the *luxCDE* genes are dispensable. Attempts have been made to clone the full *luxABCDEI* operon into a phage, although with mixed results [25, 26]. The *luxI* and *luxR* genes encode a quorum-sensing system, in which LuxI generates an acyl-homoserine-lacton autoinducer that interacts with the LuxR protein and amplifies the light-emitting reaction [27].

The bacterial luciferase enzyme catalyzes the oxidation of flavin mononucleotide ($FMNH_2$) and a long-chain aldehyde to water, FMH, and a carboxylic acid, thereby emitting light.

$$\boxed{\mathrm{FMNH_2 + O_2 + R - COH \rightarrow FMN + H_2O + R - COOH + Light\ (490\,nm)}}$$

Equation 1. Light-emitting reaction catalyzed by the bacterial luciferase

In contrast, the insect (firefly) luciferase catalyzes a two-step reaction with an unstable intermediate based on the substrate luciferin, a carboxylic acid present in many firefly species.

$$\boxed{\begin{array}{r}\mathrm{1.\ Luciferin + ATP \rightarrow Luciferyl\ adenylate + Pyrophosphate}\\ \mathrm{2.\ Lyciferyl\ adenlyate + O2 \rightarrow AMP + Oxyluciferin + Light\ (560\,nm)}\end{array}}$$

Equation 2. Light-emitting reaction catalyzed by the firefly luciferase

The luciferase is engineered to be encoded by the reporter bacteriophage under control of a promoter, which should be highly activated upon infection of the bacterial host cell. ATP and $FMNH_2$ are provided by the metabolism of the host cells. The substrates (aldehyde or Luciferin) have to be supplied exogenously. A significant difference exists between the accessibility of the substrates: whereas the aldehyde used by the bacterial luciferase readily diffuses through bacterial cell walls and membranes, the Luciferin does not. As a consequence, target bacterial cells have to be lysed either by letting the phage complete its lifecycle or by external action for the insect luciferase to gain access to its substrate. Both enzymes are reasonably active at 30 °C, but exhibit temperature-dependence.

3 Principles of Reporter Bacteriophage Construction and Use

The construction of the bioluminescent reporter bacteriophage follows one unique principle: the reporter genes are integrated in the phage genome, but due to a lack of an own metabolism, the phage particles of course remain "dark." Only when the phage infects a suitable host and the DNA transduced into the bacterium, gene expression can occur, which results in active reporter protein and light-emitting bacteria.

One intrinsic property of the phage is essential for the development of bioluminescent reporter phages: the approach harnesses the host specificity of bacteriophages, which is limited to genus or species level and sometimes even serovar or strain level. Ideally, a reporter phage should be able to target all relevant members of a pathogen group, at the desired discrimination level (species, serovar, strain), but no related organisms. In some cases, this is difficult to achieve, for example, in *Salmonella enterica* with its more than 2,500 serovars. In other cases, however, a narrow host range is desirable, such as when discriminating between pathovars of *Pseudomonas* [28]. The choice of bacteriophage is quite essential for the success of the assay. Both virulent and temperate bacteriophages are, in general, suited for the task. However, a temperate phage often displays a very narrow and limited host range, and might not be the ideal choice for detection of all members of a species. Also, prophages confer homoimmunity to similar temperate phages, which might prevent gene expression from reporter phage genomes.

Also, bacteria might become resistant to phage infection by various mechanisms, such as receptor mutation, CRISPR systems, restriction-modification systems, or abortive infection, and thus generate a false-negative signal. Although the likelihood for resistance development is low (especially in a food production setting, where potentially resistant bacteria are removed from the production chain with the food and not reintroduced), the possibility needs to be addressed. Bacteriophage resistance may be associated with a high fitness cost and therefore could be expected to be lost quickly when the selective pressure (phage) is removed [29]. Moreover, bacteriophage resistance occurs in subfractions of a bacterial population, whereas the rest of the population is still sensitive to infection and produces a detectable light signal when infected with a luciferase phage.

Another prerequisite for the construction of a reporter phage is that the phage genome is amenable to genetic manipulation. Several different approaches can be used for constructing a reporter phage. Besides transposition and homologous recombination, direct cloning is a possibility with smaller genomes of temperate phages [30]. Direct cloning requires the availability of a genetic manipulation system for the host bacterium. A genetically stable insertion of *luxAB* into a phage genome under control of a strong promoter should not negatively affect host range or infectivity or ability to multiply of the phage. An important consideration is where to place the reporter genes. In some cases, it may be possible to replace nonessential genetic regions by the reporter system, although identification of such

regions is not an easy task. Phage genes are usually densely packed on the genomes, often featuring coding capacities of more than 90 % [31–33]. Moreover, insertion of large gene fragments into noncoding regions might be difficult to achieve. Despite the recent advances in sequencing technologies and an increasing number of phage genomes available, the function of most genes in phage genomes is not clear [34, 35]. Determining a nonessential gene or a noncoding region in which a reporter gene may be inserted is therefore not trivial. This fact also represents the major difficulty in transposition-based methods for generating a reporter phage: random insertion likely results in disruption of functions required for infection and multiplication.

A further restriction to the more straightforward design and construction of reporter phages is that direct cloning is rarely possible. In the best case, chromosomally integrated temperate phages (prophages) will be amenable to genetic manipulation in the same way as the host chromosome. However, this depends on the availability of suitable tools for manipulating this organism. In contract, genetic manipulation of virulent bacteriophage chromosomes is inherently much more difficult, because the phage DNA is only accessible for modification during the infection process. The method of choice is homologous recombination and double cross-over between the mutation locus placed on a plasmid and the phage DNA, which will result in mutated DNA incorporated into new phage particles. Because the frequency of successful recombination is very low, the challenge is to isolate the recombinant virus from the background of wild-type phages. However, the desired phage can be relatively conveniently selected from the background by screening for “bioluminescent plaques” formed on infected host cells (in the presence of the substrate for the luciferase enzyme).

The reporter gene should be placed under control of a strong promoter, active upon infection of the bacterial host. Naturally, a phage late-gene promoter would be best suited for this task. Alternatively, nonphage promoters to enhance sufficient levels of reporter gene expression in certain host bacteria could also be used.

The physical structure of the phage genome plays a major role in determining the correct placement of a reporter gene. Phage genomes must be circularized or primed by proteins for efficient, loss-free DNA replication in the host cell. Therefore, most phage genomes feature overlapping single-stranded ends or terminal redundancies to enable circularization upon infection [36], the latter of which may be extensive [37]. Any genetic manipulation, in particular addition of sequence, must take into account that phage capsids provide limited space, and packaging signals must not be destroyed by gene insertion. This is well illustrated by construction of *Listeria* reporter phage A511::*luxAB*. The 2.2 kb *luxAB* gene was inserted into the 3′-region of the major capsid protein gene, which features a strong promoter and is highly expressed during phage replication [38]. At the time this work was done in 1995, no complete genome sequence and no information about the A511 genome structure was available. Only 12 years later, the complete genome sequence of A511 and the 3.125 bp fixed terminal redundancy of its chromosome were resolved [37]. These findings explained why it was possible to insert the 2.2 bp *luxAB* fusion into the A511 genome. However, it is still unclear

how the A511 terminal redundancy is generated and maintained [37]. In another example, Kuhn et al. reported severe difficulties in obtaining a transposon-mutated Felix O1 *Salmonella* reporter phage, which was attributed to the phage not being able to accommodate additional genetic information. Eventually, essential genetic information had to be deleted and supplied *in trans* to enable recombinant reporter phage generation [25].

A very important advantage of the detection of bacteria using bioluminescent phages is the fact that bacteriophages rely on the host cell metabolism for gene expression and protein synthesis, and the bioluminescence reaction also requires substrates such as $FMNH_2$ originating from an active metabolism. Consequently, luciferase and bioluminescence cannot be produced outside a viable host cells. The reporter phage assay is therefore superior to PCR or any other molecular method, which cannot distinguish between live and dead cells.

The theoretical detection limit of a reporter phage assay is a single viable cell. However, this is greatly dependent on the reporter system and detector, and specific characteristics of the phage, such as its multiplication rate and requirements. The matrix from which bacteria are isolated, enriched, and detected also plays an important role. Clearly, there is a significantly better performance of the assay in broth or buffer than directly in a food matrix [14, 29, 39]. The food surface provides hiding niches for bacterial target cells, which absorb the majority of phage particles, or inactivate them by indigenous substances. Also, free diffusion of the phage particle is essential for successful binding to the target host, a condition not given in most food materials [40]. In most cases, a pre-enrichment step is strictly required for detection of bacterial cells, with a reasonable lower limit of 10 or 100 cells per ml or g food being detectable [14, 29, 39]. With longer pre-enrichment, this limit may be lowered down to one cfu/ml/g or less [39], which is well within the regulatory limits for most foods and pathogens.

Some drawbacks of bioluminescent reporter phage assays should also be mentioned here, such as the lack of thermostability of the LuxAB fusion from *Vibrio*, which is progressively inactivated at temperatures of 30 °C and above, limiting its use to bacteria that can grow at or below this temperature (the psychotrophic pathogen *Listeria* represents an ideal case). Also, emission of bioluminescence from infected bacteria is transient, and the time window for measurement is shortly after injection of the aldehyde substrate. This is caused by the limited supply of $FNMH_2$ in infected cells, which is rapidly depleted by the bioluminescence reaction, resulting in a short burst of light emission of typically less than 20 s [15, 38]. Increasing the measurement time will therefore not result in a lower detection limit, that is, a better signal-to-noise ratio [15].

Furthermore, the use of relatively simple single-tube luminometers is not easily applicable in large-scale screening of food materials. Bioluminescence measurement from phage-infected bacteria in small liquid samples is much better suited to multiplates and semiautomated luminescence readers with substrate injection systems. However, such systems may be relatively expensive, similar to any other modern high-throughput screening method. Yet, reporter bacteriophages may also be integrated into lab-on-a-chip approaches. Bacteriophages can be immobilized

on membranes and other solid supports [19, 41–43], and the detection system could potentially be miniaturized. Hazbon et al. reported simplification of the detection of the light-emitting reaction from *Mycobacterium* reporter phages by using inexpensive Polaroid film, and achieved equal sensitivity compared to a luminometer, albeit requiring a longer detection time [20].

Finally, the lack of a suitable bacteriophage for the target organism, or difficulties in genetic manipulation might present major hurdles for development of other or additional bioluminescent phages.

4 Specific Bioluminescent Reporter Phages

This chapter provides an overview of bioluminescent reporter bacteriophages constructed for specific organisms in the past, but also discusses applications for the detection of pathogens in food and hospital settings.

Early attempts to construct recombinant bacteriophages featuring a reporter molecule for bacterial detection (not necessarily a bioluminescent marker) were undertaken by Castilho et al., who cloned a promoterless β-galactosidase gene into bacteriophage Mu. When host cells were infected, a random insertion of the lacZ gene in the host chromosome could occur, and if the insertion happened downstream of a host promoter, β-galactosidase was produced and resulted in a color reaction with an exogenous substrate, enabling cell detection [44].

A few years later, Ulitzur and coworkers modified phage lambda by introducing the bacterial luciferase genes into the bacteriophage genomic DNA, and created the first luciferase reporter phage described, termed L28 [13]. The resulting bioluminescent reporter bacteriophage could detect as little as 10 viable *Escherichia coli* cells approximately 100 min after infection [13, 45].

Salmonella was the target for several attempts to construct useful reporter phages. As one of the major foodborne pathogens with millions of cases worldwide and a very large projected economic loss, screening foods for *Salmonella* contamination is essential for food producers. Different luciferase phages were constructed, based on phages P1, P22, and Felix O1 [25, 30, 46]. Chen and Griffiths reported *Salmonella* detection in eggs, and could detect all of the 51 tested *Salmonella* isolates with no false-positives using a three-phage cocktail. The test detected 10 cfu/ml of *Salmonella* from broth with 6 h pre-enrichment, and within 24 h directly from contaminated whole eggs [47]. Another P22-based reporter phage was evaluated by Stewart and Turpin et al. in two independent studies and it was able to detect as low as 100 *Salmonella* cells [48, 49]. Thouand et al. report a P22::*luxAB* assay for detection of *Salmonella* in poultry samples, with detection limits of 10^2–10^4 cfu/ml, depending on the matrix used [50]. However, P22 is a narrow host-range phage, and thus not suited for general *Salmonella* diagnostics. In 2002, Kuhn and coworkers described another reporter phage based on the broad host-range *Salmonella* phage Felix-O1. Despite multiple attempts, the authors could not insert the *luxAB* genes by transposon mutagenesis

[25], likely because of the terminally redundant invariable genome ends of this phage (Klumpp and Marti, unpublished results). The Felix-O1 reporter was eventually made by replacement of three genes with a *luxAB* cassette, and *in trans* supply of one of the essential genes. However, the reporter phage performed poorly in strains other than the propagation host [25]. One interesting property of this specific construct is the fact that it is genetically locked; that is, it can only reproduce in the complemented laboratory host strain. Therefore, it is unable to reproduce and spread into the environment, a fact that might help address general concerns regarding use of GMO reporter phages.

In 1991, Kodikara et al. developed a reporter phage for near-online detection of enterobacteria. For proof-of-concept, an abattoir environment was used, and meat-processing surfaces were sampled. The authors reported a 10^4 cfu/g detection limit for a 1 h quick test, and could significantly reduce the limit to 10 cfu using a 4 h sample pre-enrichment [51].

E. coli, namely serotype O157:H7 was also used as a target for reporter phage detection. The phage was based upon the temperate virus ΦV10, and featured promoterless bacterial *luxAB* genes that were inserted by transposon mutagenesis on a 3.6-kb fragment [52]. The authors claimed that ΦV10 was able to detect O157:H7 after only 1 h of infection, and could detect 64 % of screened *E. coli* O157:H7 isolates. Use of a temperate phage is probably the reason for the insufficient range of detectable isolates, and construction of further reporter phages was suggested [52].

In 2008, Ripp and coworkers published the construction of an *E. coli* O157:H7 reporter based on phage PP01 [26]. Earlier, the same phage was engineered as a fluorescent reporter phage by Oda et al., to present a GFP molecule on its capsid. The phage enabled specific tagging of target bacteria, which could be seen under the fluorescence microscope [17]. Ripp et al. engineered the phage to encode the *luxI/R* quorum sensing, and a *luxCDABE* bioluminescence operon. The reporter phage is described to respond specifically and autonomously to the presence of *E. coli* O157:H7. The presence of the LuxI and R proteins serves for signal amplification. LuxI produces an autoinducer N3–(oxohexanoyl-) L-homoserine lactone (OHHL), which activates LuxR, which in turn upregulates the *lux* gene transcription. The LuxCDE gene products catalyze synthesis of the luciferase aldehyde substrate, avoiding the need for external substrate addition during detection [26]. The phage featured a detection limit of 10^3 cfu/ml from pure cultures. To push this value down to 1 cfu/ml, an enrichment step was introduced, resulting in a total assay time of approximately 10–12 h. In apple juice, the detection limit for *E. coli* O157:H7 could was 100 cfu/ml, and in water it was 1 cfu/ml. In contrast, the limit was a massive 10^6 cfu/g from ground beef, possibly due to a high background bioluminescence because of the intrinsic presence of the OHHL autoinducer in beef [26]. This points to a major drawback of signal autoamplification: the presence of an inducer-like substance can trigger a light-emitting reaction and might produce false-positive results.

Arguably one of the first reporter phages developed for Gram-positive pathogens and successfully applied to foods was *Listeria* phage A511:*luxAB* [38].

Listeria is the causative agent of the rare but severe infection termed listeriosis [53], and is almost exclusively transmitted via contaminated foods [54, 55]. Culture-based detection methods for *Listeria* take 4–6 days, which is quite problematic considering the short shelf-life of most ready-to-eat foods and delicatessen. Phage A511 is a virulent myovirus of *Listeria* and can infect the majority of *Listeria* strains [56]. It later became a model organism for the bacteriophage subfamily *Spounavirinae*, which comprises many related phages of potential biotechnological interest [37, 57]. The reporter phage was developed with the goal to reduce detection time and limits. Current regulations demand the absence of *Listeria* from 25 g of food for certain types of food, which is still difficult to confirm even by the most modern molecular detection methods. In this work, a *luxAB* gene cassette from *Vibrio harveyi* was inserted downstream of the major capsid gene of A511, and expression is driven by the dedicated and strong P_{cps} promoter, resulting in high expression levels. Recombination into the wild-type phage was achieved by plasmid-based double-crossover during phage infection and genome replication [38]. Mutant phages could be identified and isolated using the luciferase activity, and could be calculated to have occurred at a relatively high frequency of approximately 1:50,000. Maximum signal intensity is approximately 100–140 min postinfection of *Listeria* target cells [38].

In a follow-up study, the efficacy of A511:*luxAB* was evaluated in artificially and naturally contaminated foods [39]. The phage enabled highly sensitive detection of *Listeria* contamination in 55 out of 348 tested field samples, with no false-positives. A pre-enrichment step of 20 h was used to achieve best results, which is superior to the 4–6 days required for the standard plating method. Depending on the type of food, detection of 0.1 cell/g could be achieved, whereas in other, more complex types of food, such as minced meat, positive diagnostics of 10 cells/g food were possible [39]. It should be noted that A511 is not species specific, and can infect and yield signals with other *Listeria* species. This is not necessarily a drawback, inasmuch as any *Listeria* contamination is considered undesirable and a marker of poor hygiene or contaminated raw products [58].

More recently, the same concept was used to modify A511 with a different reporter system. The *celB* glycosidase from *Pyrococcus furiosus* features extreme heat stability—in contrast to the original *luxAB* system, which is instable above 35 °C—and sufficient pH stability. The enzyme is most active at 102–106 °C and pH 5–5.5, a major advantage compared to the temperature-sensitive luciferase enzyme [15]. Because of its versatile activity, featuring both β-galactosidase and β-glycosidase activity, a wide range of chromogenic, fluorescent, or chemiluminescent substrates can be used for pathogen detection. The system has been proven to be suitable for detection of *Listeria* from the food matrix, with detection limits as low as 10 cells. The assay can be fully automated to 96-well plate format [15]. One advantage of the *celB* reporter phage is that detection does not need to be carried out in a specific time window as with luciferase activity, but the color conversion of a cromogenic substrate is an end point assay and can be determined any time after incubation.

Earlier attempts to construct bioluminescent reporter phages have focused on clinically relevant pathogens such as *Mycobacterium tuberculosis*. These attempts were driven by the slow-growing nature of these bacteria and the resulting long time required for detection. The luciferase reporter phages developed for this species used firefly luciferase and were based on phages TM4, L5, and D29 [12, 59, 60]. Both D29 and L5 exhibit a relatively broad host range, infecting *M. tuberculosis*, *M. smegmatis,* and *M. bovis*. TM4 infects *M. avium, M. tuberculosis, M. smegmatis,* and *M. ulcerans*. In 1995, Sarkis and coworkers presented the L5 luciferase reporter phage for *Mycobacterium smegmatis* [12]. The temperate bacteriophage carries the firefly luciferase genes inserted into a tRNA gene region. This is in contrast to previous attempts at generating *Mycobacterium*-specific reporter phages, which utilized phage TM4 or D29 and a shuttle plasmid strategy [59, 60]. The latter experiments resulted in a reporter system with relatively poor detection capacity of 10^4–10^5 cells [12]. However, this approach is somewhat different from using a lytic phage, as the system actually depends on formation of lysogens, which then constitutively express the *lux* genes, and light output increases for many hours after infection [12]. The drawback of using the firefly luciferase is that because of the poor diffusion properties of the substrate, bacterial target cells must actually be lysed in order to generate the light signal.

Several reports have followed up on the detection of mycobacteria by bacteriophages. In recent approaches, bacteriophages are utilized to deliver a GFP or other fluorophor to mycobacterial cells, which is then used to monitor them by fluorescence microscopy or flow cytometry [61]. A study conducted in Mexico revealed a 76 % detection rate of *M. tuberculosis* from 523 sputum samples using luciferase reporter phage. In a later study, the same authors report detection of the target bacteria in 94 % of 84 contaminated sputum samples [62, 63]. Luciferase reporter phages and “fluoromycobacteriophages” have also been used for detection of drug-resistant isolates of *Mycobacterium tuberculosis* [60, 64] (see below for an extended discussion of this specific application.).

Not surprisingly, bioluminescent bacteriophages were also designed to target potential biosafety-relevant human pathogens, such as *Bacillus anthracis* and *Yersinia pestis* [65]. For *B. anthracis*, a reporter phage was developed based on the temperate, but broad host-range Wβ phage, which infects all nonencapsulated *B. anthracis* strains [66]. Most *Bacillus* ACT-group phages infect all three organisms with similar efficiency, and are thus not useful for the above purpose. In contrast, codetection of *B. thuringiensis* and *B. anthracis* would not be considered problematic for detection in foods. The *Vibrio harveyi luxA* and *luxb* were inserted into the dispensable *wp*40/*wp*41 locus, under control of an optimized *Bacillus* promoter and featuring a suitable ribosome binding site [66]. The assay yields a quick response time (first light signal only 16 min following infection), and a detection limit as low as 1,600 cfu/ml of vegetative cells. Spores may be triggered to germinate and could be detected after as low as 60 min [66]. However, use of a temperate phage has the massive drawback of not necessarily lysing the host cell. Immunity to infection can easily occur, resulting in an absence of light signal even though the target organism is present.

The same authors also produced a reporter phage for *Yersinia pestis* [67], which (in an improved version) could potentially also be used to assess antibiotic susceptibility of this pathogen. *Yersinia* typing phage ΦA1122 was equipped with the bacterial *luxAB* genes in a noncoding region, and the ability of the phage to generate a drug-concentration-dependent signal was harnessed [68].

Plant pathogenic bacteria also represent targets for the development of reporter bacteriophages, as they cause significant losses in agriculture, and early-warning and screening systems are needed. Schofield et al. report the development of phage PBSPCA1::*luxAB*, which specifically targets *Pseudomonas cannabina pv. alisalensis*, the causative agent of bacterial blight of crucifers [28]. The *Vibrio harveyi luxAB* genes were inserted into an *phoH* homologue in the phage genome, under control of a strong bacterial promoter [28]. The detection limit was 260 cfu/ml, at approximately 120 min after infection [28, 69].

4.1 Antibiotic Resistance

A simple but intriguing concept for testing of antibiotic susceptibility of bacterial isolates was proposed by Ulitzur in 1987 [13], and further developed with reporter bacteriophages for Mycobacteria. If the host cell is treated with an antibiotic to which it is sensitive, phage multiplication cannot continue, gene expression of reporter genes from the phage genome is attenuated or abolished, and cells emit less or no light. Ulitzur et al. confirmed this hypothesis by providing antibiotic-response curves for different antibiotic substances on *E. coli* infected by reporter phage λ L28 [13]. In a more recent study, Schofield et al. transferred this concept to a reporter phage for *Yersinia pestis* [68]. The bioluminescent signal generated by A1122::*luxAB* was fitness-dependent and directly linked to the drug concentration used for challenge. The phage-based antibiotic sensitivity test performed similar to the CLSI microdilution method, but yielded results after 60–120 min instead of 48 h [68]. This concept has been further exploited by reporter phages for *Mycobacterium* [60, 62, 70]. Riska et al. could detect the resistance to fast-acting drugs such as Rifampicin and slow-acting drugs such as ethambutol or ciprofloxacine within hours or 2–3 days, respectively [21]. Similar results were also obtained with myocbacterial fluorescence reporter phages [64].

4.2 Combination of Bioluminescent Reporter Phages with Other Technologies

The detection limit of bioluminescent reporter phages can be further and significantly improved if immunomagnetic separation technologies are used in pre-enrichment of target bacteria. Especially suitable seems the use of cell-wall binding domains of bacteriophage endolysins coated on paramagnetic beads for

fast and highly efficient enrichment of pathogens [71, 72]. Pre-enriching the target bacteria with magnetic separation, followed by detection with bioluminescent reporter phages could lower the detection limit by several orders of magnitude, and provide ultrafast diagnostics of viable bacteria only (Kretzer and Loessner unpublished data). The methods may be automated and performed in a high-throughput setting. Favrin et al. used classical immunomagnetic separation for this purpose, and were able to detect as low as 3 cfu *Salmonella enteritidis* per 25 g of food [73].

5 Summary, Conclusions, and Outlook

The use of bioluminescent bacteriophages for detection of foodborne pathogens offers a number of unique advantages. The system allows for rapid and highly specific detection of selected bacterial agents, and requires a minimum of equipment and training to use. Bioluminescent reporter bacteriophages can detect only viable bacteria, as the reporter gene expression relies on an active host metabolism. The assays are fast, highly specific, and generally inexpensive. Despite these clear advantages, the reporter bacteriophage technology is not as widely accepted or used as one would imagine. One potential drawback of the reporter phage construction is the requirement of a sufficiently specific but broad host-range bacteriophage. If none is available, it must be isolated from the environment, often a laborious and lengthy process, and not always crowned by success. Especially in closely related organisms such as *Salmonella*, *Shigella*, and *E. coli*, phages specific for one but not the other organisms are difficult to find [74]. It may also be challenging to achieve appropriate genetic manipulation of the selected phages without affecting the phage lifecycle or ability to infect its host. However, with the increased availability of genome sequences of bacteriophages and bacterial hosts due to novel DNA sequencing technologies, the further development of reporter bacteriophage platforms might gain momentum, especially if difficult-to-detect bacterial species are targeted. Last, but not least, coupling of efficient enrichment and immobilization technologies with bioluminescent bacteriophage assays provides the microbiologist with powerful diagnostic tools for specific detection of bacterial pathogens.

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Part IV
Applications of Bioluminescence in Health

Application of Enzyme Bioluminescence for Medical Diagnostics

Ludmila A. Frank and Vasilisa V. Krasitskaya

Abstract Nowadays luciferases are effectively used as analytical instruments in a great variety of research fields. Of special interest are the studies dealing with elaboration of novel analytical systems for the purposes of medical diagnostics. The ever-expanding spectrum of clinically important analytes accounts for the increasing demand for new techniques for their detection. In this chapter we have made an attempt to summarize the results on applications of luciferases as reporters in binding assays including immunoassay, nucleic acid hybridization assay, and so on. The data over the last 15 years have been analyzed and clearly show that luciferase-based assays, due to extremely high sensitivity, low cost, and the lack of need for skilled personnel, hold much promise for clinical diagnostics.

Keywords Bioluminescence · Ca^{2+}-regulated photoprotein · Diagnostics · Immunoassay · Luciferase · Nucleic acid hybridization assay

Abbreviations

Ab	Antibody
Ag	Antigen
ATP	Adenosine-5′-triphosphate
BRET	Bioluminescence resonance energy transfer
CMV	Cytomegalovirus
ELISA	Enzyme-linked immunosorbent assay
EYFP	Yellow fluorescent protein
GFP	Green fluorescent protein
hCG	Human chorionic gonadotropin

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hFSH	Follicle stimulating gonadotropic hormone
hGH	Human growth hormone
hGLuc	Highly bright Gaussia luciferase
hLH	Luteinizing gonadotropic hormone (lutropin)
JAK2	Janus kinase 2
LIPS	Luciferase immunoprecipitation system
LNA	Locked nucleic acid
MBL	Mannose-binding lectin
miRNA	Micro RNA
mRNA	Messenger RNA
MTB	*Mycobacterium tuberculosis*
NASBA	Nucleic acid sequence-based amplification
PAP	Prostatic acid phosphatase
PCR	Polymerase chain reaction
PEXT	Primer extension
PSA	Prostate specific antigen
RLuc	*Renilla* luciferase
RT-PCR	Reverse transcription polymerase chain reaction
SNP	Single nucleotide polymorphism
TLR4	Toll-like receptor 4
TSH	Thyroid stimulation hormone (thyrotropin)

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1 Introduction

At present, among bioluminescent systems the best-known are those derived from fireflies, marine bacteria, coelenterates, and copepods. “Best-known” here implies availability of cDNAs coding corresponding light-emitting proteins and affordable

recombinant proteins, chemically synthesized substrates, information on 3D structure, and mechanism of light-producing reaction. A high quantum yield of the reactions and a high sensitivity of modern photometers make possible the detection of luciferases down to the attomole. The foregoing provides the basis for a variety of analytical applications of luciferases: from environment monitoring to detection of different events in living cells. In this chapter, we focus on bioluminescence application in in vitro binding assays that play an increasingly important part in medical diagnostics. The data presented demonstrate a successful use of luciferase reporters in immunoassay, nucleic acid hybridization assay, simultaneous detection of several targets in one sample, and carried out in solid-phase and homogeneous formats.

2 Immunoassay: State-of-the-Art

Immunoassay is a diverse group of analytical techniques used throughout clinical laboratories. Since 1959 when immunoassay principles were expounded by Yalow and Berson there has been an exponential growth in the range of applications and in the number of novel and ingenious assay designs. Regardless of the application and underlying technology the assay involves four components: the antigen (Ag) to be detected, the antibody (Ab) used for detection, the method to separate the antigen–antibody complex (Ag–Ab) from unbound reactants (in the case of heterogeneous assay), and the method for Ag–Ab complex detection. The most popular and simple to understand type of immunoassay is the immunometric (sandwich) solid-phase design (Fig. 1a). In this case, the surface is activated with antibody (Ab) that captures test antigen from the sample and the antibody Ab (tracer), specific to another part of the antigen and labeled with a signal generation molecule or isotope. After incubation resulting in Ab–Ag–Ab complex formation the unbound labeled antibody is washed away. The final stage of the assay involves the measurement of signal level, which in this type of analysis is proportional to antigen concentration in the sample.

When the target analyte is small molecules (certain hormones, drugs, etc.), the type of immunoassay applied is different. In this case, only one antibody and the target analyte labeled with a suitable signal generation material are used as the key reagents. The sample analyte competes with the labeled one for binding with antibody on the surface (competitive immunoassay). In this type of assay the signal level is indirectly proportional to antigen concentration in the sample (Fig. 1b).

Of less use is the so-called homogeneous immunoassay that does not require a separation stage for unbound labeled antibody. The signal is generated by the tracer only on binding with analyte. Under specific design of the assay the complex formation quenches the signal completely.

A wide variety of immunoassay designs and formats has been developed over the years (see [1]). In general the efficacy of any immunoassay depends on two

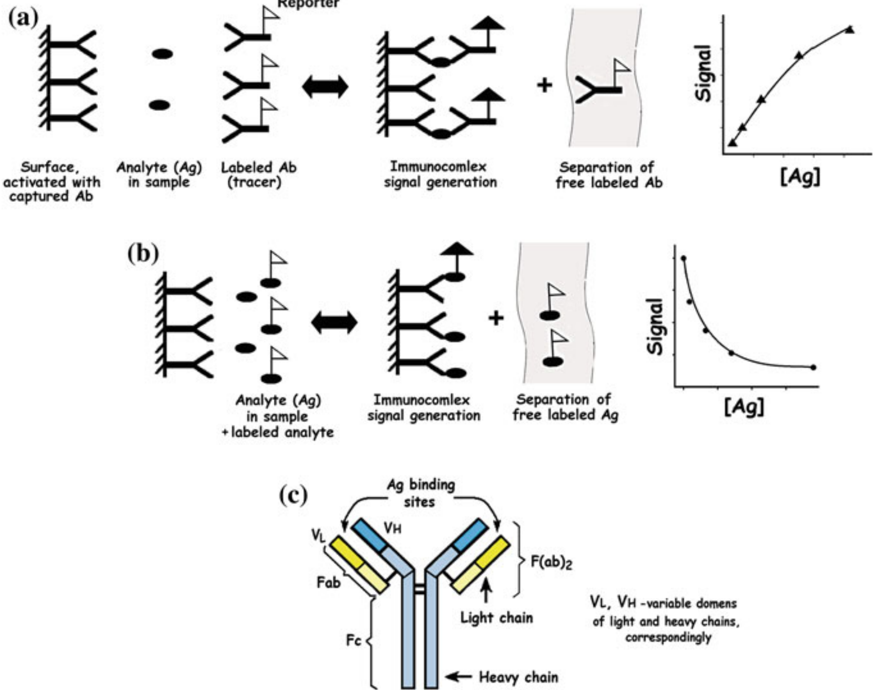

Fig. 1 Scheme for: **a** sandwich-type and **b** competitive immunoassays, and **c** basic antibody structure (human IgG) demonstrating pairs of identical heavy (*blue*) and light (*yellow*) chains. Both pairs contain variable domens, V_H (*dark blue*) and V_L (*dark yellow*), responsible for antigen binding

factors: the efficiency of the immunocomplex formation and the ability of the detection system to register this complex with high sensitivity. Complex formation is provided by antibodies' specificity and binding affinity to the antigen. Antibodies are proteins, which are produced in animals by immunological response to the presence of a foreign substance (Fig. 1c schematically presents a basic antibody structure). Antibodies are a crucial component of immunoassay performance due to their ability to bind to an extremely wide range of natural and man-made molecules, cells, and viruses; exceptional specificity for the analyte that enables us to assay it in complex biological media (sera, urine, etc.); and the strength of binding, providing the formation of a strong noncovalent complex Ag–Ab that survives at processing and signal generation.

The immunocomplex detection system (signal generation plus measuring tool) accounts for immunoassay sensitivity. The prime requirement for the one is the detection of the label above background noise. A wide range of labels and their nature-dependent detection systems are applied in immunological assay. Radiolabels are the first to be used but the problems dealing with radioactive wastes and inherent instability of radiolabeled reagents have stimulated the development

of nonradioactive labels. The enzymes catalyzing chemical reactions producing visual signals (e.g., color or light) are now used more frequently as compared to other labels. Enzymes may be detected at very low concentration because a single enzyme molecule catalyzes many reactions without being consumed. Thus the signal and consequently the assay sensitivity increase by several orders in contrast to a label that produces just one signal event. To become a tracer, the enzyme is covalently linked to antibody or antigen (depending on the assay format). The other conjugation method involves a molecular biology technique in which a gene encoding enzyme and gene encoding biospecific polypeptide (antigen, antibody, etc.) are joined in one frame. Translation of this fusion gene yields a single polypeptide with properties of both biospecific and reporter modules. The main shortcoming of enzyme-based assay is that enzymes and substrates may be unstable and require special conditions to maintain activity (pH, temperature, cofactors, absence of inhibitors, etc.). To suit as a label, any enzyme should fulfill some more conditions: availability, stability to chemical modification and under storage, simplicity of use, and lack of toxicity. The search for enzymes meeting the above requirements is being continued. Nowadays luciferases are the point of interest in this view because a high quantum yield of bioluminescent reaction and capacities of modern photometers make possible their detection down to the attomole. So they are excellent reporters for a great variety of analytical applications. The perspectives of application of the presently known luciferases as labels for immunoassay are further considered.

2.1 *Luciferases as Labels for Immunoassay*

Bacterial luciferases are relatively big (around 80 kDa) heterodimeric proteins. They catalyze long-chain aldehyde oxidation and need reduced flavin, which can be provided by NADH:FMN-oxydoreductase. The system is too complicated to be applied as a reporter in immunoassay.

Firefly luciferases are single-chain polypeptides with a molecular mass around 60 kDa, catalyzing ATP-dependent luciferin oxidation. The bioluminescent reaction demonstrates the highest quantum yield (48 %, [2]) among those known today. Therefore as little as 0.02 pg of the protein (subattomolar level!) can be measured. Luciferases' cDNAs from some fireflies (e.g., *Photinus pyralis*, *Luciola mingrelica*) were cloned; the recombinant analogues were obtained and are now commercially available products. The enzymes and their genetically modified improved analogues have been applied as genetic reporters in molecular biology and for ATP detection in microbiology. But due to firefly luciferase instability to chemical conjugation with Ab or Ag, its application as a label in immunoassay looked unreasonable. Nevertheless the firefly luciferase-based labels were obtained using gene-fusing technology. The luciferase gene was fused with DNA, coding biotin-acceptor polypeptide and expressed in *Escherichia coli* cells. A chimeric protein fully retained luciferase activity and also was efficiently biotinylated

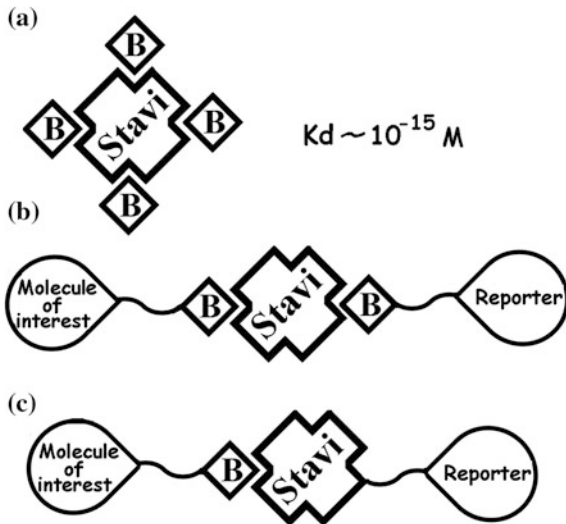

Fig. 2 Biotin-streptavidin/avidin system (**a**), the strongest noncovalent biological interaction known (kd $\sim 10^{-15}$ M) and one of the most widely used affinity pairs in molecular, immunological, and cellular assays. Biotin (*B*) is introduced into the molecule of interest and into reporter, and streptavidin (Stavi) with four biotin-binding sites serves as a strong and specific bridge between these molecules (**b**). Sometimes, conjugates streptavidin-reporter are used (**c**)

in vivo. Using biotinylated luciferase in combination with streptavidin and biotinylated antibodies, the sensitive immunoassays of hCG, human growth hormone (hGH), TSH, PSA, and staphylococcal protein A have been developed [3–5]. The principle of how the biotin–streptavidin system works is shown in Fig. 2.

Another technique applies the auxiliary label enzyme, acetate kinase or pyruvate phosphate dikinase [6]. These enzymes being stable to chemical modifications yield ATP as one of the reaction products. The auxiliary enzyme conjugated with antibody forms the immunocomplex with a target and its activity is determined by measuring the amount of produced ATP using intact firefly luciferase.

A big group of luciferases responsible for bioluminescence of different marine animals (soft corals *Renilla reniformis* and *Renilla muelleri*, copepods *Metridia longa* and *Gaussia princeps*, jellyfish *Aequorea victoria*, hydroid polyp *Obelia longissima* etc.) catalyzes oxidation of the same substrate molecule, coelenterazine. (One may see its structure in Fig. 3.) The cDNAs of all the luciferases listed were cloned, their recombinant analogues obtained and are now commercially available, as well as coelenterazine and its chemical derivatives.

Renilla luciferases (RLuc) are single-chain polypeptides with a molecular mass around 36 kDa. With genetic modifications applied, their analogues with essentially improved properties regarding thermal stability, light output, or color were obtained [7, 8], thus making them more suitable as reporters for any kind of assay. Nevertheless the essential loss of RLuc activity chemically conjugated with the other molecules is observed [9]. A highly sensitive bioluminescent immunoassay

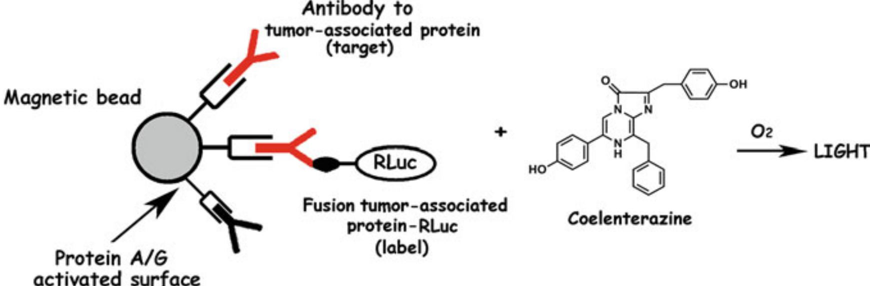

Fig. 3 Luciferase immunoprecipitation system. Immunocomplex antibody to tumor-associated protein: fusion of this protein-RLuc is captured by magnetic beads, covered with protein A/G. The bioluminescent signal arises with substrate (coelenterazine) addition. Protein A/G: recombinant fusion protein that combines IgG (Fc fragment) binding domains of proteins A and G

was developed using the luciferase genetically fused with antigene or antibody. Figure 3 displays the luciferase immunoprecipitation system (LIPS), developed by Burbelo and coauthors to detect antibodies responsible for tumor-associated proteins [10], and a variety of infectious agents [11–13]. Briefly, this approach involves: (a) construction of antigen–RLuc fusion and expression of the fusion protein in mammalian COS cells; (b) incubation of RLuc–antigen fusion and analyzed sera sample to bind antibody of interest; (c) immobilization of the complex RLuc–antigen fusion–antibody on protein A/G beads, and (d) quantitation of antigen-specific antibody by adding coelenterazine and measuring light production. The most important point of this technology is the mammalian production of fusion protein to provide correct folding and specific posttranslational modifications of the antigenic domain, which are impossible under bacterial expression. Of course it makes the analysis more expensive, although in some crucial cases it is justifiable by high sensitivity.

Fusion proteins RLuc–antibody have mostly been engineered for ex vivo or in vivo analytical applications. For these constructions, the variable regions V_L and V_H chains of immunoglobulin (Fig. 1c) being minimal engineered antibody fragments, capable of efficient target binding, are usually used as luciferase partners. Under bacterial expression, periplasmic secretion of chimeric protein is usually employed in order to provide antibody domain affinity. As shown for anticarcinoembryonic antigen antibody–RLuc fusion proteins [14], bacterial expression allowed purification of small amounts of proteins, with only about half of them recovered intact, whereas under mammalian expression the recovery was greater, and owing to the lack of proteases, the protein purified remained stable for several months when stored at 4 °C.

Luciferases from *M. longa* and *G. princeps* are single-chain polypeptides (20–24 kDa) that contain 10 Cys residues, organizing several disulfide bonds. The bacterial expression gives mostly unfolded proteins and the obtaining of an active protein requires a special refolding procedure or expression in eukaryotic cells.

These luciferases are unstable to chemicals, too: *Metridia* luciferase chemically conjugated with biotin loses almost 70 % of bioluminescent activity [15, 16]. *Gaussia* luciferase is the smallest native luciferase yet discovered (19.9 kDa). Its biotinylated active derivative was obtained using gene-fusing technology such as biotinylated firefly luciferase and applied to detect a biotinylated model DNA fragment through streptavidin bridge [17].

Luciferases from marine ostracods *Vargula hilgendorfii* and *Cypridina noctiluca* are highly homologous single-chain glycosylated polypeptides (around 62 kDa) containing 34 Cys residues. They are very stable (50-h half-life time at 37 °C) possibly due to the formation of several Cys–Cys bridges. Recently the recombinant *Cypridina* luciferase expressed in yeast was chemically conjugated with small haptenes: biotin or prostaglandin E2 with a loss of activity exceeding 70 % [18–20]. Despite this, these derivatives displayed high sensitivity in interferon alpha and prostaglandin E2 immunoassay.

Both types of marine luciferases from copepods and ostracods are secreted proteins and, in accord with numerous publications, are successfully used as secreted reporter enzymes for continuous in vivo and ex vivo monitoring without cell destruction, whereas their application in in vitro analytical systems is a rare case.

Luciferases of a peculiar kind are Ca^{2+}-regulated photoproteins responsible for bioluminescence of a number of marine coelenterates such as jellyfish *A. victoria* and *Clytia gregaria*, and hydroid polyps *O. longissima* and *Obelia geniculata*, among others.

Ca^{2+}-regulated photoproteins are stable complexes of apophotoprotein (single-chain polypeptide with a molecular mass around 20 kDa) and the preoxidized substrate molecule peroxycoelenterazine, which is strongly but noncovalently immobilized in the protein hydrophobic cavity [21–23]. The primary structures of photoproteins reveal high homology and all have three Ca^{2+}-binding sites. Upon Ca^{2+} addition, proteins undergo conformational changes resulting in coelenterazine decarboxylation. The products of the reaction are CO_2, coelenteramide, and a flash of blue light. In contrast to the luciferase reaction, photoprotein bioluminescence does not depend on oxygen or substrate concentration. Bioluminescence is detected in a luminometer allowing injection of calcium solution and simultaneous measurement of light. The cDNAs of the proteins were cloned, expressed in bacterial cells both in a soluble form and as inclusion bodies, and isolated with a high yield. Recombinant apoproteins were effectively activated with a synthetic coelenterazine under calcium-free conditions in the presence of O_2 and a reducing reagent producing photoprotein of high activity (close to that of a natural protein) without folding problems. Simple technology of expression and purification provides 15–20 mg recombinant photoprotein of high purity and activity per 1 g of bacterial cells. Regarding estimating photoproteins as labels, several obvious advantages of those should be taken into account: availability of recombinant proteins; high quantum yield of the reaction and virtual absence of the background signal making the photoprotein detection down to an attomole; practically unlimited linear range of bioluminescence; the simplicity of the reaction trigger;

tolerance to chemical modifications, and conjugation with the other molecules. The conjugates in solution as well as in frozen and lyophilized states were found to be stable and well-stored. A great number of publications described photoprotein-based immunoassay of different analytes of clinical interest: hormones, interleukins, oncomarkers, and the like, and infections (see [24]). The assays were carried out in different formats (sandwich, competitive), and performed in solid-phase, homogeneous, and even in flow injection variants. The papers consider investigations of model samples as well as sera, saliva, and mucous of patients and experimental animals. Clinical trials began in 1994. At that time aequorin-based thyrotropin (TSH) assay (developed and marketed by SeaLite Inc., USA) was tested in the Pathology and Laboratory Medicine Departments of Emory University and Veterans Administration Hospital (Atlanta, USA) [25]. The authors measured serum TSH in 153 euthyroid individuals with thyroidal illnesses (primary hypo- and hyperthyroids, thyroid cancer, etc.) applying bioluminescent methods and compared the results with the data obtained by commercially available Nichols, ACS-180 and TOSOH methods. The aequorin-based method was found to have the required performance characteristics (e.g., the functional sensitivity of 0.017 mIU/L, which is higher than in the cases of ACS-180 and TOSOH assays) and is "clearly qualified as a last, third-generation TSH assay."

From the above presented data it appears that the application of classical luciferases as reporters in immunoassay is complicated by their instability to chemical conjugation with biospecific molecules (recognition elements) or by the problems of genetically fused proteins' folding under their bacterial expression. By contrast, Ca^{2+}-regulated photoproteins are free of these shortcomings and this makes them perfect in vitro reporting molecules. The immunoassay on their basis is fast and simple and, what is most important, highly sensitive in detecting the target.

3 Nucleic Acid Hybridization Assay Based on Bioluminescent Reporters

Nucleic acid hybridization is based on the ability of individual single-stranded nucleic acid molecules to form double-stranded molecules (i.e., to hybridize to each other) in accordance with complementarity of bases. Standard nucleic acid hybridization assays involve the formation of heteroduplexes between labeled single-stranded nucleic acid probes and complementary sequences within a target nucleic acid. In recent years, bioluminescent proteins have found rapidly expanding application as effective labels in DNA/RNA assays, in clinical diagnostics as well. The polymerase chain reaction (PCR) has proved to be of great value in diagnostic research. Its ability to amplify specific nucleic acid sequences several millionfold has facilitated detection of a small number of DNA copies. For some diagnoses it is necessary to establish the presence or absence of the sequence of interest (diseases associated with infectious agents, gene deletions, SNP, etc.).

But more often, quantitative PCR analysis is important for evaluation of therapy effectiveness, detection of gene expression through reverse transcriptase-polymerase chain reaction (RT-PCR), and so on.

Many strategies have been developed to quantitate PCR products. The traditional approach of calculation is visualization by DNA band density after separation on agarose gel, or scintillation counting of radiolabeled products. The obvious shortcomings of these techniques—low sensitivity, bulkiness to perform, or necessity to use radioactive materials—hamper implementation of these assays in routine settings. The assay improvement was achieved by using automated capillary electrophoresis and laser-induced fluorescence [26]. The need for electrophoresis was avoided by using magnetic beads coated with streptavidin to capture biotinylated PCR fragments [27]. After hybridization with a hapten-labeled probe, these beads were analyzed either by flow cytometry or by immunoenzymatic assay based on chemiluminescent, fluorescent, or colorimetric labels. Quantitative realtime PCR assay allows continuous monitoring of accumulation of PCR products during the amplification reaction. This provides identification of the cycle of near-logarithmic PCR product generation (threshold cycle) and, by inference, the relative quantification of the template DNA present at the start of the reaction. Because the amplification products are monitored in realtime as being formed cycle by cycle, no postamplification handling is required. The absolute quantification is performed according to either an internal standard coamplified with the sample DNA, or to an external standard curve obtained by parallel amplification of serial known concentrations of a reference DNA sequence. However, realtime PCR has certain disadvantages such as the high cost of the equipment and requirements for high technical skills. Despite the use of high temperatures, nonspecific primer annealing may occur during PCR, and errors will be exponentially amplified through multiple cycles. In order to discriminate these products and to obtain a higher level of specificity, a hybridization step should be included in assays.

Several methods have been developed for quantitating PCR products using a microplate format. The tag can be attached to the DNA during the PCR reaction or via a labeled probe. The PCR product is immobilized on a surface using biotin–streptavidin, digoxigenin–antidigoxigenin, direct chemical conjugation, or other methods. The DNA is then detected with a colorimetric, fluorescent, or luminescent tag. The obvious advantages of microplate assays are low cost, fewer steps, and the possibility to handle a large number of samples simultaneously. However, in the desirable exponential PCR reaction phase, the concentration of a product is often too low to be detected by standard methods. This technical hurdle may be overcome using sensitive detection methods.

In recent years, bioluminescence has found rapidly expanding application in DNA/RNA assays. With no excitation required, bioluminometric assay offers higher detectability, wider linear range, and much simpler instrumentation than fluorometric methods. A variety of hybridization assays has been published, most of them describing the use of Ca^{2+}-regulated photoproteins as reporters. The first reports describing the use of recombinant aequorin as a reporter molecule in a

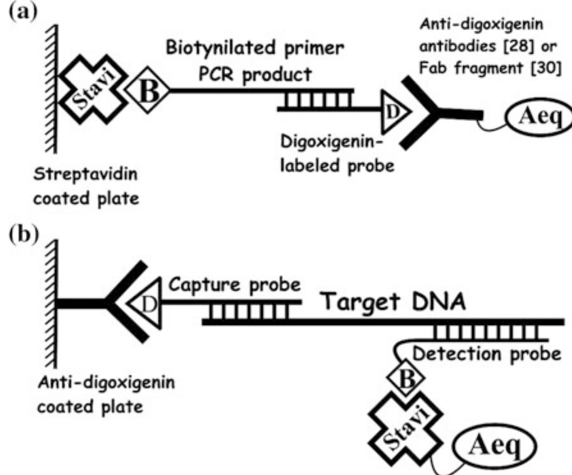

Fig. 4 Bioluminometric nucleic acid hybridization assays with recombinant aequorin as a reporter proposed by **a** Xiao [28] and Siddigi [30], and **b** Galvan [29]. *D*, digoxigenin, *Aeq*, aequorin

bioluminometric nucleic acid hybridization assay appeared in 1996 [28–30] (Fig. 4). By now photoproteins are applied as reporters in the assay of infectious agents; quantitation and detection of gene expression during infectious disease; detection of cancer markers, microRNAs, and SNP; quantification of the allele burden at oncogenic somatic point mutation. Some examples for illustration are considered below.

3.1 Bioluminescent Assay of Infectious Agents

Early detection and recognition of pathogens is extremely important for effective treatment and prevention of epidemic diseases. The aequorin-based hybridization assays are well suited to detect clinically important agents, specifically when pathogens are either difficult to isolate or propagate. In the case of tuberculosis, conventional plating techniques require approximately 4 weeks of cell growth in an approved high biosafety facility prior to enumeration of colon-forming units (CFU). A reverse transcription-PCR (RT-PCR) assay was developed by Actor for rapid identification of *Mycobacterium tuberculosis* (MTB) infection in tissues from murine models. Tissue samples were collected, reverse transcribed, and amplified in the presence of biotinylated 16 s rRNA MTB specific primer sequences. Amplicons captured on streptavidin-coated wells were coupled with a digoxigenin labeled probe, and detected using an aequorin-conjugated antidigoxigenin antibody. The assay allowed detection of the infection within 24 h after tissue was obtained [31]. In a similar system, malaria pathogen following infection of mice with *Plasmodium berghei* was detected within blood cells after a one-day exposure [31].

The extremely high sensitivity of pathogen detection using bioluminescence has been highlighted in an assay of cytomegalovirus (CMV) within patient serum samples [31]. In this case an oligonucleotide probe was directly labeled with aequorin. As few as 4 copies of CMV genome template were detected in less than 1 h.

Guenthner and Hart developed a quantitative competitive PCR assay of the human immunodeficiency virus Type 1 (HIV-1) DNA or RNA. The test is based on the presence of a competitive internal standard containing an internal 80-bp deletion of HIV-1 gag target sequence. Using a primer pair in which one primer is biotinylated, PCR amplicons are bound to a streptavidin-coated microplate, denatured, and probed with a digoxigenin-labeled wild-type or internal-standard probe. The hybridized probes are detected with antidigoxigenin antibody–aequorin conjugates. Results indicated high sensitivity with a quantifiable range of 100–10,000 copies of HIV gag [32].

Song et al. have developed a nucleic acid sequence-based amplification (NASBA) assay for amplification of *Chlamydia trachomatis* 16S rRNA and coupled it with a microtiter plate bioluminescent assay employing aequorin for detection and quantitation. NASBA products were hybridized with a biotinylated capture probe and fluorescein-labeled detection probe. The capture probe was immobilized on a streptavidin-coated plate. The hybrids were detected by an aequorin antifluorescein conjugate. This assay can detect 1,000 molecules of 16S rRNA providing a sensitivity equal to or greater than one genomic equivalent; it is quantitative through four logs of bacterial load and may be useful for large-scale epidemiological studies aimed at understanding the relationship between variables such as antigen load, serotype, and transmission rates [33]. In a similar study, Coombes et al. have developed a NASBA-based bioluminescent assay of *Chlamidia pneumonia* RNA, which may be applied toward elucidation of the role of differentially expressed chlamydial genes during infection and disease pathology. They used NASBA amplification techniques coupled with bioluminescent readout and were able to quantitate ompA RNA over nearly an 8-log linear range, beginning at approximately 100 ompA RNA molecules. In addition, the sensitivity of detection was tenfold greater than Northern blotting and detection using chemiluminescent-labeled probes [34].

Doleman and colleagues developed a bioluminescence DNA hybridization assay to detect the most deadly species of malaria, namely, *Plasmodium falciparum*. Of great interest is that the assay does not require PCR amplification of the sample and is based on the competition between the target DNA (analyte) and the biotinylated DNA for hybridization (probe) on the microplate, which is detected by the streptavidin–aequorin conjugate signal. This bioluminescence hybridization assay demonstrated a detection limit of 3 pg/μL and was employed to detect the target DNA in standard and spiked human serum samples. This assay is potentially suitable for multiplex analysis, even in laboratories that lack sophisticated equipment [35].

3.2 Bioluminescent Assay in Oncology Diagnostics

Molecular diagnostics is an essential analytical tool in oncology. It implies diagnosis of cancer of inherited type, identification and diagnosis of molecular biomarkers that aid in early detection, and prediction of responses to therapies, tests for cancer risk, and so on. Many methods have been developed for cancer diagnostics including the ones with bioluminescent readout.

Galvan and coauthors developed a bioluminescence hybridization assay combined with reverse transcriptase polymerase chain reaction to detect mRNA for prostate-specific antigen (PSA) [29]. Because metastatic prostate cancer calls for a therapeutic intervention that differs from that applied to organ-confined disease, the detection of circulating PSA-expressing cells becomes a unique test for correct disease staging. The authors used aequorin as a reporter molecule for a microtiter well-based hybridization assay (Fig. 4b). PSA mRNA from a single cell, in the presence of one million cells that do not express PSA, was detected, with a signal-to-background ratio being 2.5. Typical variation coefficients obtained were 6 %. The configuration of the proposed assay does not require labeling of PCR products during amplification. The amplified DNA is determined by simultaneous hybridization with capture and detection probes. The applicability of the assay is not limited to PCR products but extends to any target DNA for which two specific oligonucleotide probes can be synthesized.

The need for early molecular markers and their detection methods in cancer diagnosis is tremendous. Recent studies revealed that miRNA mutation or misexpression correlate either positively or negatively with various human cancers. miRNAs are considered as useful early diagnostic and prognostic cancer markers, candidates for therapeutic intervention, and targets for basic biomedical research. However, methods for highly sensitive and rapid detection of miRNA directly from cells that would serve as a suitable diagnostics platform are lacking. Cissell et al. have developed an assay for detection of microRNA, miR21 in breast cancer cells using *Renilla* luciferase as a label [36]. The miR21 was linked to several cancers. The levels of miR21, for instance, were found to be elevated in breast, liver, ovarian, pancreatic, and brain tumors compared to corresponding normal tissues [37, 38]. Here, RLuc was conjugated to an oligonucleotide probe complementary to the miR21 sequence. A competitive assay was set up between miR21 from the sample and RLuc-labeled miR21 probe for binding with the biotinylated anti-miR21 oligonucleotide probe (Fig. 5). The duplexes obtained were immobilized on the streptavidin-activated surface. The signal was measured after the addition of RLuc substrate coelenterazine and was correlated with the amount of free miR21 in the sample. The hybridization assay was developed in a microplate format with a total assay time of 1.5 h and without the need for sample PCR-amplification, thus making it more suitable for application in clinical diagnostics. The optimized assay allowed a detection limit of 1 fmol. The method is applicable for sensitive, accurate, and precise measurement of miR21 in vitro and ex vivo.

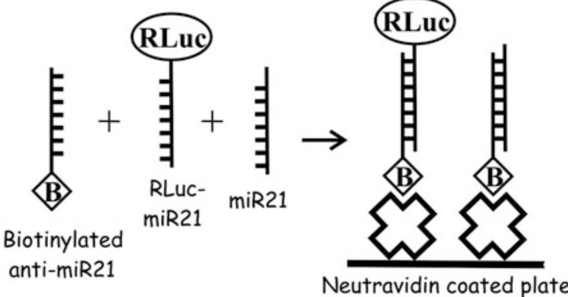

Fig. 5 Scheme for bioluminescence-based hybridization assay developed for detection of miR21 [36]

The need for detection of minority mutation (i.e., a few mutants within a high excess of wild-type alleles) arises frequently in the field of cancer genetics. Routine tumor biopsies often consist of an inhomogeneous mixture of stromal cells plus tumor cells encompassing a wide range of genetic profiles and mutations. Quantification of the mutant allele burden (percentage of the mutant allele) is critical for diagnosis, monitoring of therapy, and detection of minimal residual disease. Detection of minority mutation is often important in mitochondrial disease and mutation/polymorphism screening of pooled DNA from many individuals. With point mutations, the challenge is to quantify the mutant allele while discriminating from a large excess of the normal allele that differs in a single base-pair. Tsiakalou et al. reported the first bioluminometric assay for quantification of the allele burden and its application to JAK2 V617F somatic point mutation [39]. This mutation is known to be strongly associated with the myeloproliferative disorders polycythemia vera and essential thrombocythemia [40]. The proposed method is performed in microtiter wells and involves a single PCR for amplification of both alleles, followed by primer extension (PEXT) reactions with allele-specific primers (three cycles). The products are captured in microtiter wells and detected by oligo(dT)-conjugated photoprotein aequorin. The authors demonstrated that the luminescence signal from the mutant allele is linearly related to the allele burden. As low as 0.85 % of the mutant allele can be detected and the linearity extends up to 100 %. The assay is complete within 50 min after the amplification step. The method can be applied to a large number of reported somatic mutations where quantification of the allele burden is required.

Iliadi et al. developed two bioluminescent methods that enable absolute quantification of the allele, with this mutation as an example [41]. The first method exploits the ability of a nonextendable locked nucleic acid (LNA) effectively to inhibit the PCR-amplification of the normal allele while the amplification of the mutant allele remains unaffected. The second method employs allele-specific PCR primers, thereby allowing the amplification of the corresponding allele only. In both assays, absolute quantification of the mutant allele is achieved through coamplification of the recombinant DNA internal standard (DNA competitor). The amplified products from the target and internal standard are quantified by a hybridization assay performed at microtiter wells and exploiting the advantages of

the aequorin reporter. The ratio of luminescence values for target DNA and DNA competitor linearly relate to the number of JAK2 V617F allele copies initially present in the sample. The methods allow absolute quantification of less than 300 copies of the mutant allele even in samples containing less than 1 % of the mutant allele. The novelty and advantages of the proposed method are: (a) the concept of competitive PCR is exploited for the absolute quantification of the mutant and normal alleles in single-point mutations using properly designed recombinant DNA internal standards; (b) the combination of the above principle with a high-throughput and highly sensitive bioluminometric assay; (c) the considerably lower cost of instrumentation and reagents compared to other methods, such as realtime PCR and BEAMing technology; and (d) incorporation of LNA probes in the competitive PCR simplifies the methods because it does not require two allele-specific primers.

Bioluminescence-based systems for detection of nucleic acids appeared to be a powerful and flexible analytical tool for accurate and rapid assay. In the case of photoproteins, sufficient assay sensitivity allows the detection of amplified product before the linear relationship of the target to product is lost, as well as the direct detection of low copies of unamplified target. The use of such analytical systems considerably improves the assays in terms of simplicity and costs.

4 Multianalytical Bioluminescent Assay

The multianalyte approach allows intensification of the assay procedure especially when the proper diagnostics require simultaneous detection of several analytes in one sample. Both immuno- and molecular assays of the kind were developed using either different bioluminescent reporters or luciferases in tandem with the other reporters.

Ito et al. [42] developed a highly sensitive and rapid immunoassay of two antigens involving aequorin and firefly luciferase labels. The authors detected two couples of antigens: prostatic acid phosphatase (PAP) with PSA or PSA with alphafetoprotein (AFP). As tracers, chemical conjugate aequorin–antidigoxigenin Fab fragment and biotinylated in vivo firefly luciferase were applied. A Ca^{2+} injection triggered flash-type aequorin bioluminescence and then the mixture of luciferin, ATP and Mg^{2+} was placed into the wells to initiate luciferase bioluminescence. With the developed technique applied, PAP, PSA, and AFP were detected in standard and then in clinical sera. The values obtained from the developed simultaneous assay were in good correlation with those derived from conventional methods.

Two kinds of biotinylated in vivo firefly *Luciola lateralis* luciferases with different bioluminescent spectra maxima—559 nm and 607 nm—were applied for simultaneous assay of pepsinogen I and pepsinogen II [43]. To divide signals, several optic filters were applied, but the high signals' overlap and long way to form immunocomplexes made the assay rather bulky.

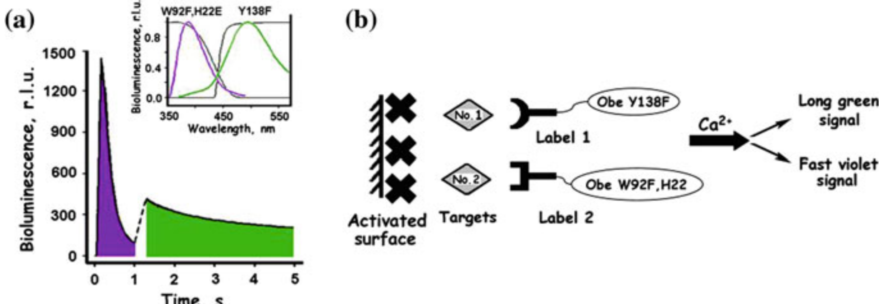

Fig. 6 Dual analyte single-well bioluminescence assay based on photoprotein obelin variants with substantially altered bioluminescence spectra and kinetics. **a** Bioluminescence of mixture of obelins transmitted through filter I (*fast violet signal*) and filter II (*slow green signal*); *dashed line*, time for filter replacement. *Upper inset*, bioluminescence spectra of obelins (*colored lines*) and optical filter transmission (*black lines*). *r.l.u.*, relative light units. **b** Scheme for simultaneous solid-phase immunoassay of two targets

With site-directed mutagenesis applied, photoprotein obelin mutants with substantially altered bioluminescence spectral and kinetic characteristics were obtained: W92F, H22E emitting fast ($kd = 0.6\ s^{-1}$) violet signal ($\lambda_{max} = 387$ nm) and Y138F with slow ($kd = 6.1\ s^{-1}$) greenish light ($\lambda_{max} = 498$ nm) with small spectral overlapping [44]. Using those as reporters, a dual analyte single-well bioluminescence assay was developed based on the spectral and time signal resolution (Fig. 6). The approach was successfully applied to simultaneous immunoassay of: (a) total and IgG-bound prolactins [45]; (b) gonadotropic hormones, luteinizing (lutropin or hLH) and follicle stimulating (hFSH); (c) gene allelic variants at SNP genotyping of the human F5 gene encoding Factor V Leiden polymorphism 1691 G → A (R506Q) [46]. Many clinical samples were investigated and the obtained results were in good agreement with those obtained by traditional techniques.

Tannous et al. proposed dual analyte bio/chemiluminometric method for simultaneous genotyping of IVS-1-110 locus of the human globin gene in a single microtiter well [47]. They used aequorin and alkaline phosphatase as reporters with consequent triggering of their bio/chemiluminescence reaction. The principle of the approach is illustrated in Fig. 7. Genomic DNA, isolated from whole blood, was first subjected to polymerase chain reaction using primers flanking the polymorphic site. A single oligonucleotide-ligation reaction employing two allele-specific probes, labeled with biotin and digoxigenin, and a common probe carrying a characteristic tail was then performed. When the probes perfectly matched their target sequence, they were joined covalently by ligase, whereas a mismatch at the junction inhibited ligation. The ligation products were captured in a microtiter well through hybridization of the tail with an immobilized complementary oligonucleotide and were detected by adding a mixture of the streptavidin–aequorin complex and antidigoxigenin-alkaline phosphatase conjugate. The aequorin was

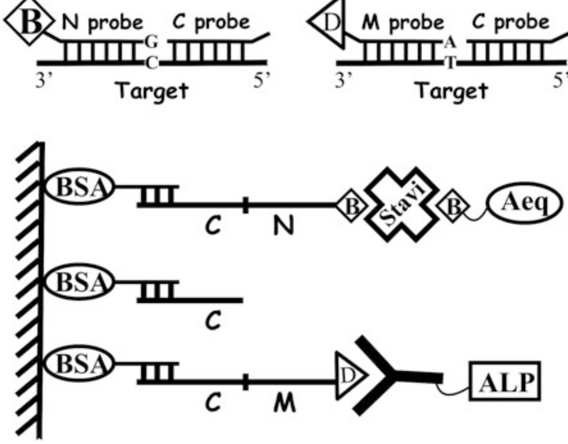

Fig. 7 Principle of bioluminometric SNP genotyping by oligonucleotide ligation reaction. *N probe* is specific for normal allele, *M probe* for mutant allele, and *C probe* is a common probe. *BSA*, bovine serum albumin; *ALP*, alkaline phosphatase

measured first by adding Ca^{2+}, then the wells were washed and the chemiluminescence of bound alkaline phosphatase was measured after incubation with substrate. The ratio of the obtained luminescence signals gives the genotype of each sample.

The same dual-analyte bio/chemiluminometric assay but for detection of PEXT reaction products was applied by Konstantou et al. for two SNPs of human mannose-binding lectin (MBL2) gene (−550 and −221) and one SNP of cytochrome P450 gene CYP2D6 (CYP2D6*3) [48]. MBL2 is a key component of the innate immune system, and its deficiency is associated with the increased susceptibility to various infections and autoimmune disorders. The CYP2D6 is important because it is involved in the metabolism of many commonly prescribed drugs [49]. PCR-amplified DNA fragments that span the SNP of interest are subjected to two PEXT reactions using normal and mutant primers in the presence of digoxigenin-dUTP and biotin-dUTP. The primers perfectly complementary with the target DNA are extended by DNA polymerase thus forming digoxigenin-labeled or biotin-labeled products that are then mixed and analyzed by dual bio/chemiluminometric assay. The proposed method provides good discrimination between the two alleles. Patient genotypes showed 100 % concordance with direct DNA sequencing data.

Elenis et al. developed a method of simultaneous genotyping of two common SNPs within the toll-like receptor 4 (TLR4) gene, that is, A896G and C1196T [50]. The method consists of a single PCR of the region spanning both polymorphic sites, followed by a quadruple PEXT reaction in a single tube. Biotinylated nucleotide is incorporated into extended primers. All four products are captured on streptavidin-coated microtiter wells and detected with a combination of four reporters, aequorin, and alkaline phosphatase, β-galactosidase, and horseradish peroxidase. Bio/chemiluminescence of each reporter was measured sequentially after washing the wells and incubation with a corresponding substrate. For each SNP, 46 individuals were genotyped. The accuracy of this method was confirmed

by sequencing. The proposed quadruple-allele bio/chemiluminometric approach provides an accurate, simple, rapid, and reproducible method for high-sample–throughput SNP genotyping.

5 Bioluminescent Binding Assay in Homogeneous Format

Homogeneous format of any assay simplifies detection by circumventing immobilization, incubation, and washing steps that are required in both ELISA and DNA hybridization assays. The detection in this case is performed due to emergence (or quenching) of the signal during the formation of specific complexes. This kind of format is essential for high-throughput assay in vivo and ex vivo, for discovering and investigation of macromolecules' interactions, protein activity, and the like. (See for reference, the exhaustive review [51]). The subject is considered in this volume in another chapter. Nevertheless preliminary investigations on the development of the assay design are carried out on simple in vitro examples and moreover there are some communications on homogeneous assays in real physiological samples. Here we just briefly dwell on some principal points.

There are two general approaches for homogeneous bioluminescent binding assay based on BRET phenomenon or on split-protein reassembly technology (Fig. 8). BRET is a naturally occurring phenomenon resulting from radiationless energy transfer between a bioluminescent donor and fluorescent acceptor [52, 53]. The efficiency of the process is known to depend on overlapping of donor luminescence and acceptor absorption spectra and on the distance between the proteins. The last one must be around 10 nm and is determined by the presence and effectiveness of molecules' biospecific interactions including antigen–antibody, ligand–acceptor, and complementary oligonucleotides. The BRET-based immunoassay principle is illustrated by hen egg lysozyme analysis [54] (Fig. 8a). The pair of *Renilla* luciferase (donor) yellow fluorescent protein (EYFP, acceptor) was applied as a reporter in a noncompetitive homogeneous immunoassay based on antigen-dependent reassociation of antibody variable domains. Two himeric proteins, an antibody heavy-chain variable fragment, luciferase (V_H-RLuc) and antibody light-chain variable fragment, yellow fluorescent protein (V_L-EYFP) emit correspondingly blue bioluminescent (max = 475 nm) and yellow fluorescent (max = 525 nm) signals. The addition of antigen induces dimerization of the two proteins and tethering of RLuc and EYFP. As a result an effect of energy transfer between bioluminescent donor (RLuc) and fluorescent acceptor (EYFP) arises and the additional yellow peak is observed in the blue-centered luciferase's spectrum. The luminescence ratio of 525/475 nm depends on the antigen concentration and is used for its evaluation. A more effective pair for BRET-based assay was developed recently, with a highly bright *Gaussia* luciferase variant (hGLuc) as a donor and highly photostable red fluorescent protein tdTomato as an acceptor [55]. The hGLuc is more suitable inasmuch as its emission (max = 470 nm) is far from that of tdTomato (max = 580 nm) and overlaps well with the excitation of tdTomato (max = 554 nm). The pair gave rather persistent

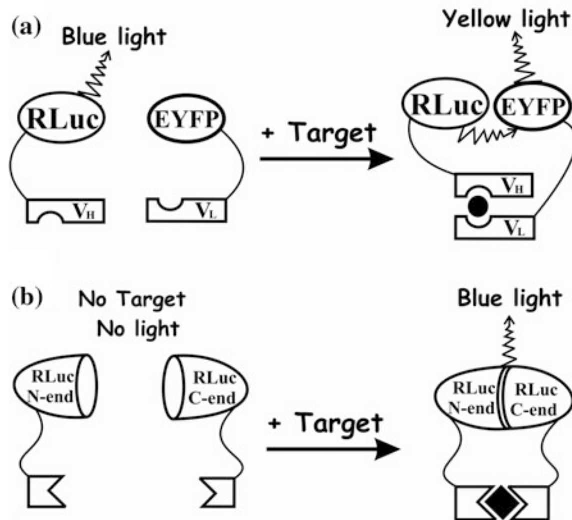

Fig. 8 Two general approaches for homogeneous bioluminescent binding assay: **a** based on BRET phenomenon and **b** on split-protein reassembly technology. *RLuc*, *Renilla* luciferase; *EYFP*, yellow fluorescent protein

signals for different buffers and selected pHs, and is insensitive to complicated sample matrices such as serum. The pair has a large luminescence spectra separation (110 nm) and provides high assay sensitivity. Instead of GFP the quantum dots are used as energy acceptors in nucleic acid hybridization assays [56, 57] or synthetic fluorochromes such as Cy3 or Cy3.5 [58].

In split-protein analytical systems, two polypeptides serve as biospecific recognition elements attached to the fragmented reporter. The protein domains interact with the desired target resulting in a ternary complex that drives the reassembly of the split-protein reporter recovering its activity [59] (Fig. 8b). For successful creation of a split reporter, a few criteria must be met: each reporter fragment by itself should not exhibit any activity, the affinity of fragments in the absence of the target should be negligible, and the reassembled split-protein must provide an easily measurable readout. This strategy has been successfully used in reassembling several luciferases as reporters, from *R. reniformis* [60], firefly [61], *G. princeps* [62], and photoprotein aequorin [63]. Here are only several examples but in reality, the range of biological activities and processes that can be monitored with a bioluminescence readout using split-protein reassembly both in in vitro and in vivo settings is unprecedented.

6 Summary, Conclusions, and Outlook

Bioluminescent proteins have been intensively used as high sensitive reporters in all kinds of binding assays including immunoassay, protein–protein and protein–ligand assays, and nucleic acid hybridization assays. They are distinctly divided

into two groups: luciferases and Ca^{2+}-regulated photoproteins. Photoproteins are resistant to chemical modifications and this makes the synthesis of highly active conjugates with different biospecific molecules to be applied as the labels possible. By the very nature of photoproteins, the bioluminescent signal of these labels does not depend on oxygen and substrate and practically always correlates with the label concentration linearly. Luciferases, by contrast, are not stable and the labels based on those are usually obtained by genetic fusing. The problems with proper folding of the fused proteins under bacterial expression require the involvement of eukaryotic expressing cells (mammalians, insects) that are rather expensive in terms of routine application. It should be noted, however, that the advantage of luciferase-based labels is the ability to increase the assay sensitivity by accumulating the signal at the substrate excess. In principle, luciferases seem more suitable reporters for studies ex vivo and in vivo. As to Ca^{2+}-regulated photoproteins, they are also used for in vivo investigations as unexcelled probes in tracing intracellular calcium fluxes.

The proteins with altered properties (stability, luminescence spectra, signal kinetics, etc.) were obtained with the help of site-directed mutagenesis. The novel variants obtained provide the development of multianalytical systems aimed at simultaneous detection of several targets in one sample. This approach benefits the assay in terms of price and time, and excludes the mistakes of separate measurements.

A high sensitivity of bioluminescent labels promotes miniaturization of the assay, that is, elaboration of microfluid and nanochip technologies and portable light-detection devices. The fastest response of these labels provides the fastest detection of analyte which is very important for cases of urgent diagnostics. Owing to portability, low cost, and the lack of need for skilled personnel, such analytical systems hold much promise for applications in clinical diagnostics.

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