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Hormetic mechanism of sulfonamides on *Aliivibrio fischeri* luminescence based on a bacterial cell-cell communication

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HIGHLIGHTS

- Sulfonamides present time-dependent hormetic effects on bioluminescence.
- Sulfonamides act on bacterial quorum sensing communication to evoke hormesis.
- LitR protein in quorum sensing system has two active forms.

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ABSTRACT

Hormesis is a biphasic dose-response model with low-dose stimulation and high-dose inhibition. The mechanism for hormesis remains inconclusive, although it is becoming a central concept in toxicology. In this paper, the hormetic mechanism of sulfachloropyridazine (SCP) on *Aliivibrio fischeri* (*A. fischeri*) luminescence was explored by investigating into the interference of SCP with the bacterial quorum sensing (QS) communications. It was revealed that the SCP-induced hormesis on luminescence was due to its action on LitR – a key protein that connects *lux* and *ain* QS communications in *A. fischeri*. It was suggested SCP acted on LitR proteins to change its active forms, which subsequently induced hormetic effects on *luxR* (QS signal receptor) and thereby the luminescence. It is the first time that the hormetic mechanism based on bacterial QS was proposed, which provides a novel insight into the essence of the hormesis on *A. fischeri* luminescence.

1. Introduction

Hormesis is a biphasic dose-response model with low-dose stimulation and high-dose inhibition, which may have great implications for all the disciplines that utilize the dose-response concept (Calabrese and Baldwin, 2003; Calabrese, 2004), e.g. the environmental risk assessment. To date, various mechanisms for hormesis have been proposed, among which the receptor-based theory and overcompensation are most prevailing (Calabrese, 2015c, 2015a, 2015b). However, these hypotheses could be only applied to part of the hormetic phenomena while fail to explain the other cases. Therefore, it is necessary to further study the hormetic mechanism and broaden its connotation with a broader view.

The luminous bacterium, *Aliivibrio fischeri*, has been widely used for the rapid toxicity detection of chemicals, based on a bioluminescence inhibition test (Backhaus et al., 1997; Parvez et al., 2006). It was revealed that the bioluminescence is regulated by the bacterial quroum sensing communication, through which the bacteria use its own language (small signaling molecules) to communicate (Henke and Bassler, 2004). Based on the bacterial QS

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communication, a new hypothesis for hormesis was proposed in a previous research (Deng et al., 2012), in which a typical hormetic phenomenon was observed with sulfonamides (SAs) on *A. fischeri* luminescence. It was assumed that SAs at low doses could competitively combine with the QS signal receptor LuxR, resulting in more LuxR production due to overcompensation, and eventually stimulate the luminescence (Deng et al., 2012); while at higher doses, SAs exhibit inhibition on the bacteria through binding to the target protein dihydropteroate synthase (DHPS) (Achari et al., 1997). This assumption could well explain the dose-dependent features of hormesis and provided a new insight into the mechanistic study of hormesis; however, this assumption was obtained from the results through computer simulation and thus needs further experiment to testify its authenticity.

In this work, we imitated a communication with *A. fischeri* by exogenously adding the QS signals (Lupp and Ruby, 2005)–N-3-oxohexanoyl-L-homoserine lactone (C6HSL) and N-octanoyl-L-homoserine lactone (C8HSL) to the SAs-exposed bacteria. C6HSL and C8HSL are two QS signals in *A. fischeri*, which are both involved in the light emitting (see details in Fig. 1). By analyzing the responses of *A. fischeri* under various exposure conditions, we expected to find out the hormetic mechanism of SAs towards bacterial luminescence. The purpose of this study is as follows: (1) to examine the time-dependent hormetic effects of SAs on *A. fischeri*, including the bioluminescence and gene expressions; (2) to figure out the SAs-induced hormetic mechanism based on bacterial QS communications.

2. Materials and methods

2.1. Chemicals and organism

The following reagents were purchased from Sigma (St Louis, MO) and used without repurifying (purity≥99%): sulfameter (SM), sulfamonomethoxine (SMM), sulfadoxine (SDX), sulfapyridine (SPY), sulfamerazine (SMR) and sulfachloropyridazine (SCP). The freeze-dried marine bacterium *A. fischeri* ATCC7744 was supplied by Institute of Microbiology, Chinese Academy of Sciences (Beijing PRC). It was reconstituted and maintained on agar slants at 4 °C.

2.2. Bacterial inhibition assay

The bacterial inhibition test was performed via 96 well microplate assays. Firstly, the bacteria were incubated in 5 ml culture medium at 22 °C for 12 h (logarithm phase), and then diluted until the light intensity reached 20 000. The diluted bacteria were added to the 96 well plates that contained the test chemicals with a series of concentrations, and incubated at 22 °C for 24 h. The bioluminescence were determined every 2 h by Mithras LB 940 Multimode Microplate Reader (Berthold). (n = 3 for control and test groups).

2.3. RNA-extraction and reverse transcription

Special precautions were taken to avoid the degradation of RNA by the ubiquitously present RNases, namely a separate working place only for RNA, separate pipettes, RNase-free plastic ware and the regular changing of gloves. Unless otherwise indicated, the whole swabs were used for the RNA extraction. The total RNA was purified with Trizol and 1 μg of total RNA was used for the reverse transcription with random primers (Invitrogen) and SuperScript III (Invitrogen).

2.4. Real-time PCR amplification

Quantitative PCR was performed on a CFX Connect Real-Time PCR System (Bio-Rad) with SYBR green detection PCR Mastermix (Bio-Rad). A three-stage RT-PCR amplification reaction was performed under the following conditions: 95 °C for 5min followed by 40 cycles of 95 °C for 30s, 55 °C for 30s and 72 °C for 30s. At last, denaturing step was used to generate the dissociation curves to verify amplification specificity. Table S1 lists the primers used in this study.

2.5. Statistical analyses

The statistical analyses were performed by Graphpad Prism software. After a Kolmogorov-Smirnov test, a one-way analysis of variance (ANOVA) was used to determine the differences among treated and control groups, followed by Dunnett test. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, which are labeled with *, ** and ***, respectively.

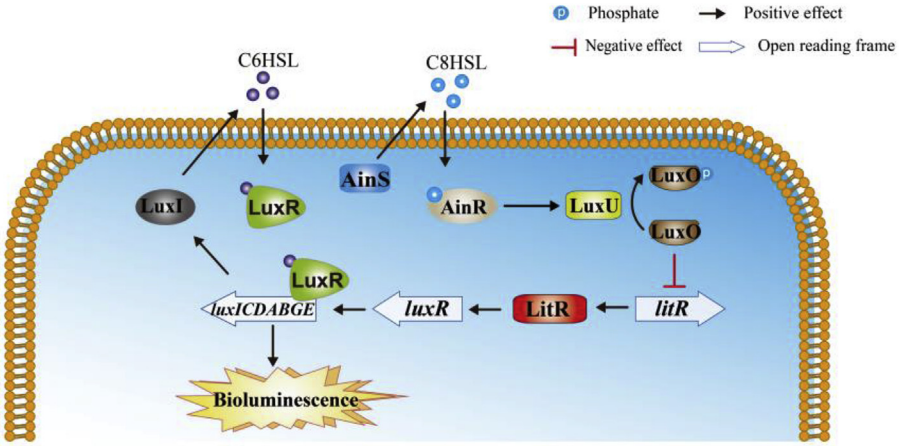

Fig. 1. The two QS systems in A. fischeri. Two QS communications have been identified that are responsible for the luminescence. One is the lux system mediated by C6HSL, and the other is the ain system mediated by C8HSL. In lux system, the bacterial language C6HSL is produced by LuxI and binds to the transcriptional activator LuxR. The LuxR-C6HSL complex then binds to the lux promoter and induces the transcription of luxICDABEG, which subsequently evokes the light emitting. In ain system, a second language C8HSL is produced by AinS. C8HSL, on the one hand, can bind to LuxR to regulate the expression of lux genes when the bacteria is at low density (but its capacity of regulating the luminescence is weaker than C6HSL); on the other hand, C8HSL can combine with AinR to stimulate LuxU and subsequently catalyze the dephosphorylation of phosphorylated LuxO (LuxO-P) to generate LuxO, as a result, the litR expression was enhanced to produce more LuxR, compensating for the deficiency of the lux system.

3. Results

3.1. Hormetic responses of A. fischeri luminescence to exposure of SCP, QS signals and their combination

SAs are among the most commonly used antibiotics, which act by competitively binding to DHPS and inhibiting the bacterial growth. Fig. S1 shows the time-dependent impacts of SCP on the bacterial proliferation, indicating a gradually increasing inhibition over time and concentration. The luminescence of *A. fischeri* is a QS-regulated behavior that depends on the bacterial density (Fig. S2); therefore, the response of the luminescence was supposed similar to that of the bacterial proliferation. But according to the results, *A. fischeri* luminescence showed a vastly different response to SCP exposure. As seen in Fig. S3, the *A. fischeri* luminescence was stimulated by SCP at the early stage (<16 h), which at 12 h showed a typical hormetic feature, with a maximum response of 150% of control at 5.4×10^{-6} mol/L (Fig. 2A). The stimulation on the bioluminescence was also observed with other SA drugs, as shown in Fig. 3.

As expected, the exogenous C6HSL and C8HSL caused remarkable stimulation on *A. fischeri* luminescence (Fig. 2B and C, also see Figs. S4 and S5). Especially for C6HSL, the stimulation could reach over 300,000 times of the control at 8.0×10^{-9} mol/L at 12 h (Fig. 2B). It should be noted that the light emitting of the bacteria only occurs when the signal molecules accumulate to a certain threshold (Fig. 1). So the exogenously adding C6HSL and C8HSL at the initial stage ($t = 0$) will certainly result in an earlier time for the occurrence of the light emitting. Therefore, the C6HSL- and C8HSL-induced hormesis could be explained by the earlier time at which the light emitting occurred than the control.

The responses of *A. fischeri* luminescence to the joint exposure of SCP and the signals were also examined. For this test, the exogenous C6HSL and C8HSL at fixed concentrations (2.1×10^{-9} mol/L and 2.4×10^{-10} mol/L) (Fig. 2 and S6) were mixed with a series of concentrations of SCP (9.2×10^{-7} -1.5×10^{-5} mol/L), respectively. According to Fig. 2D and E, the combination of SCP with the signals induced even greater stimulation on *A. fischeri* luminescence than the single C6HSL or C8HSL, which implied that the SCP-induced hormesis should be independent of the C6HSL and C8HSL. In addition, the stimulation increased with the SCP concentration, which were different from the inverted U shaped responses as induced by single SCP (Fig. 2A).

3.2. Hormetic responses of QS-related genes to exposure of SCP and QS signals

The responses of the QS-related genes, including *luxR*, *litR*, and *dhps*, were determined upon exposure to SCP (9.2×10^{-7}-1.5×10^{-5} mol/L) for 12 h, the results were shown in Fig. 4 (see Fig. S7 for more information). It was found that SCP at low concentrations (9.2×10^{-7}-2.5×10^{-6} mol/L) induced significant stimulation on *luxR* levels (Fig. 4A), which firstly increased and then decreased with SCP concentration, manifesting a typical hormetic characteristic. This response was in consistence with the response of the luminescence, which suggested that the SCP-induced hormesis on *A. fischeri* luminescence was likely due to its hormetic effects on *luxR* expression. As for *litR* and *dhps*, only inhibition was observed (Fig. 4B and C). The similar responses of *litR* and *dhps* to SCP implied a potential link between the two genes. It was reported that LitR is a central regulator of symbiosis and luminescence for the bacteria (von Bodman et al., 2008). It could control the acetate metabolism of the bacteria, and thus may affect the bacterial growth. Therefore, LitR is likely to regulate the *dhps* expression, which could probably explain the same responses of *litR* and *dhps*.

The influences of the exogenous C6HSL and C8HSL on *luxR*, *litR* and *dhps* genes were also examined. According to the results, the two QS signals, C6HSL and C8HSL affected the genes in the same way. Specifically, *luxR* (Fig. 4D and G) and *litR* (Fig. 4E and H) were stimulated in a concentration dependent manner. This was in consistence with the responses of the luminescence to C6HSL

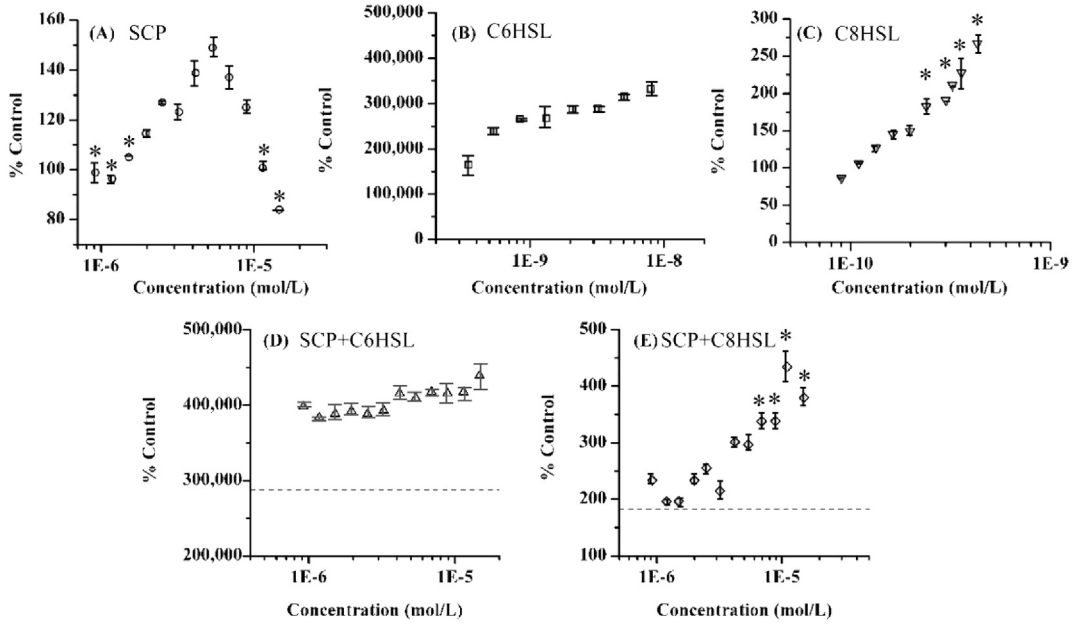

Fig. 2. Responses of *A. fischeri* luminescence to 12 h exposure of (A) SCP, (B) C6HSL, (C) C8HSL, (D) combination of SCP and C6HSL, and (E) combination of SCP and C8HSL. For the combination test, C6HSL and C8HSL at fixed concentrations (2.1×10^{-9} mol/L and 2.4×10^{-10} mol/L) was mixed with SCP at 9.2×10^{-7} -1.5×10^{-5} mol/L, respectively. The x axis in (D) and (E) indicates the SCP concentration, while the dash line indicates the response induced by single C6HSL and C8HSL. Data were presented as mean ± standard deviation, and the significant differences were labeled with *, **, and ***, which indicated $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

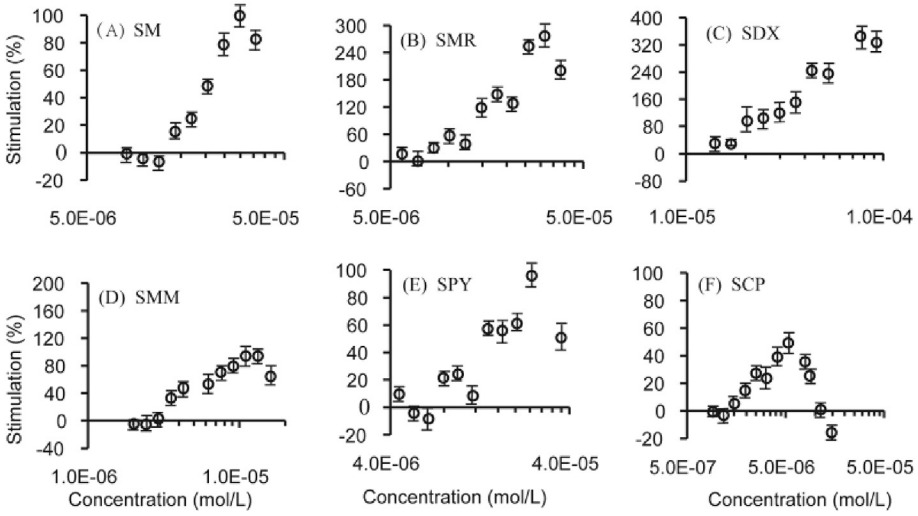

Fig. 3. Hormetic responses of *A. fischeri* luminescence after 12 h exposure to SAs. (A) SM (B) SMR (C) SDX (D) SMM (E) SPY (F) SCP. Data were presented as mean ± standard deviation.

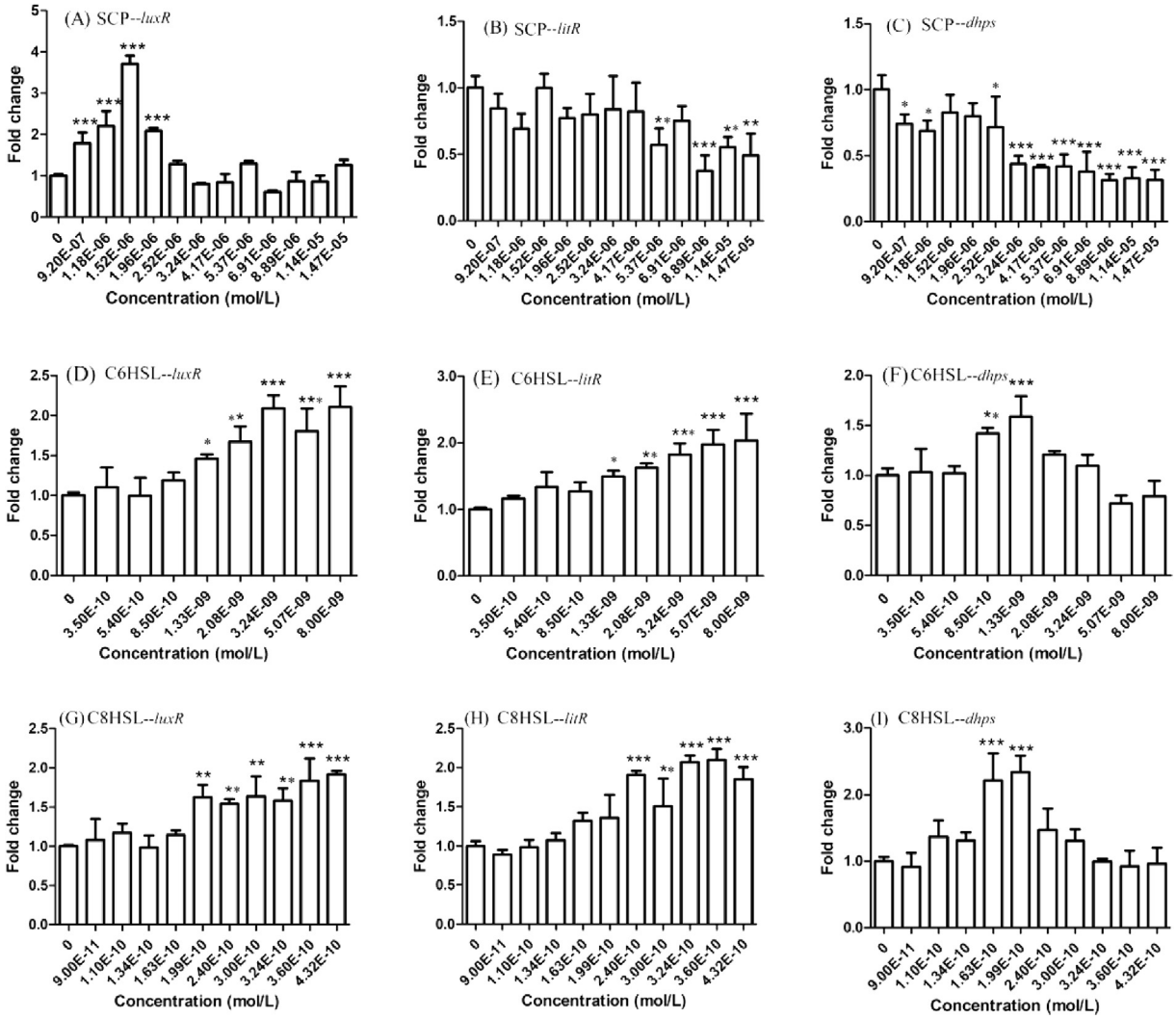

Fig. 4. Responses of *luxR*, *litR* and *dhps* to exposure (12 h) of SCP, C6HSL and C8HSL. (A) *luxR* to SCP, (B) *litR* to SCP, (C) *dhps* to SCP, (D) *luxR* to C6HSL, (E) *litR* to C6HSL, (F) *dhps* to C6HSL, (G) *luxR* to C8HSL, (H) *litR* to C8HSL and (I) *dhps* to C8HSL. Data were presented as mean ± standard deviation, and the significant differences were labeled with *, **, and ***, which indicated $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

(Fig. 2B) and C8HSL (Fig. 2C). But interestingly, *dhps* was affected in a different manner and showed a hormetic characteristic. As shown in Fig. 4F and I, *dhps* was stimulated at moderate concentrations by C6HSL and C8HSL; while at lower and higher concentrations no stimulation was observed. Since DHPS is essential for the bacterial growth, the impact on the *dhps* would naturally lead to a corresponding effect on the bacterial proliferation. This was supported by the results in Figs. S8 and S9, which show the time-dependent effects of the exogenous QS signals on the bacterial proliferation. As mentioned above, LitR is likely to regulate the *dhps* expression. But upon exposure to the QS signals, *litR* and *dhps* showed different responses. One possible explanation is that LitR at high amount may lose its activity in regulating the *dhps* expression. This could well explain why *dhps* expression increased with *litR* at lower *litR* amount while decreased with *litR* at higher *litR* amount.

3.3. Hormetic responses of QS-related genes to joint exposure of SCP and QS signals

The QS-related genes were also examined for their responses to the joint exposure of SCP and QS signals. As is similar to the experiment with the luminescence, C6HSL and C8HSL in the mixtures were fixed at defined concentrations (2.1×10^{-9} mol/L and 2.4×10^{-10} mol/L, respectively), while SCP was set at the indicated concentrations.

According to the results (Fig. 5), in the presence of the QS signals, SCP presented no effect on *litR* expression (Fig. 5B and E), but a concentration-dependent stimulation on the *luxR* and *dhps* expressions (Fig. 5A, C, D and F). According to Fig. 4C, SCP alone at the test concentration did not stimulate the *dhps* expression. Thus, the stimulation by the mixtures should be caused by the interaction between SCP and the QS signals. As shown in Fig. 4F and I, C6HSL at 2.1×10^{-9} mol/L and C8HSL at 2.4×10^{-10} mol/L would resulted in a high amount of LitR, which may lead to a decrease in the *dhps* expression. The addition of SCP to the system did not change the LitR amount, but seemed to activate the LitR at higher amounts, leading to an increase in the *dhps* expression with the SCP concentration.

4. Discussion

As shown in the Fig. 1, C6HSL and C8HSL are therefore deemed as the two languages for *A. fischeri*, which play important roles in regulating the luminescence. So when trying to explore the hormetic mechanism of SCP towards the *A. fischeri* luminescence, we delivered the exogenous C6HSL and C8HSL to the bacteria under various exposure conditions. We established a communication with *A. fischeri* by using its own languages – C6HSL and C8HSL, the observed responses of the luminescence and the QS-related genes under various conditions seem like the feedback from the bacteria. By analyzing these responses, we were expecting to reveal the hormetic mechanism of SCP.

Fig. 6 illustrates the experiments with SCP, QS signals and their combinations, and reveals the hypothetical mechanisms. As shown in Fig. 6, the individual SCP and C6HSL both could stimulate the bioluminescence. For C6HSL, the hormesis could be explained by the earlier time at which the light emitting occurs. Because the luminescence could not be observed until 11 h in control groups (Fig. 6A); while in the C6HSL-treated groups (Fig. 6B), the luminescence was observed at a much earlier time (around 4 h). While for SCP, it seems like that SCP stimulated the bioluminescence through a hormetic effect on the *luxR* expression (Fig. 4A). This could well explained why the combination of SCP and C6HSL resulted in a synergetic effect on the bioluminescence (Fig. 2D). That is, SCP increased the amount of LuxR proteins, providing more targets for C6HSL and thus increasing the bioluminescence.

The results with individual C8HSL came out different from those with C6HSL. It was found that C8HSL was likely to stimulate the *litR* expression (Fig. 4H), which subsequently increased the *luxR* expression (Fig. 4G) and the bioluminescence (Fig. 2C).

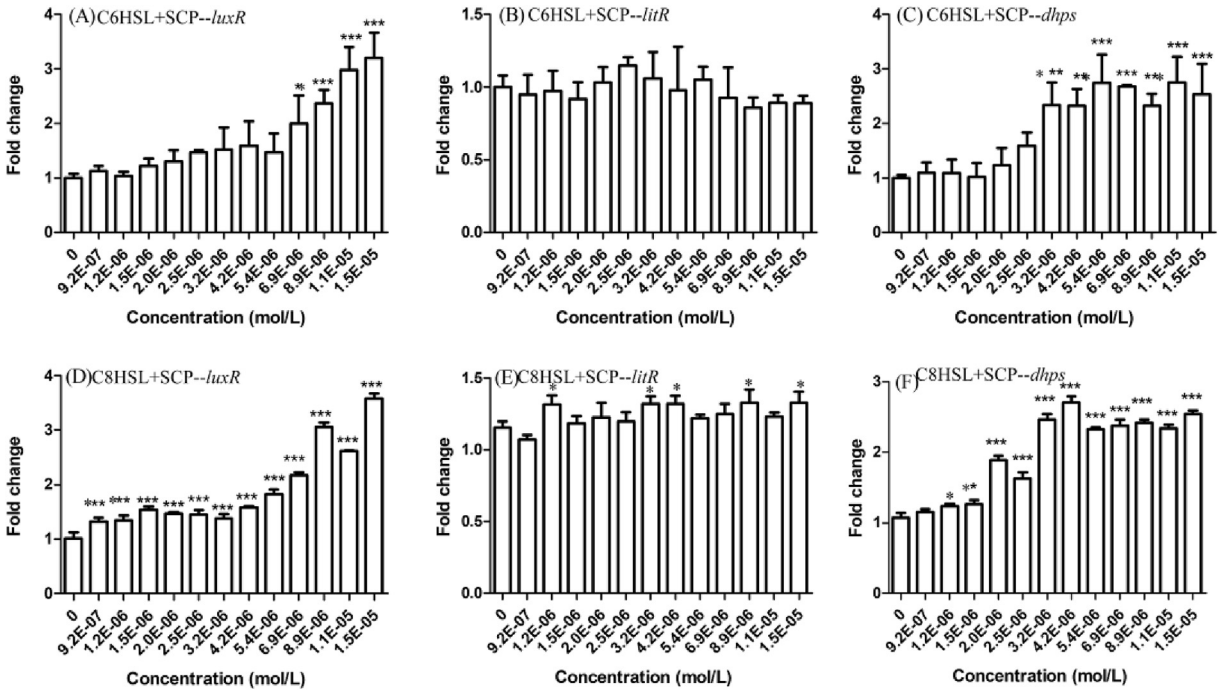

Fig. 5. Responses of *luxR*, *litR* and *dhps* expression to the mixtures of SCP and QS signals. (A)–(C): C6HSL and SCP mixtures exposure for *luxR*, *litR* and *dhps*, respectively; (D)–(F): C8HSL and SCP mixtures exposure for *luxR*, *litR* and *dhps*, respectively. The concentrations of C6HSL and C8HSL were set at 2.1×10^{-9} mol/L and 2.4×10^{-10} mol/L, respectively, while the SCP concentrations were set at the indicated values.

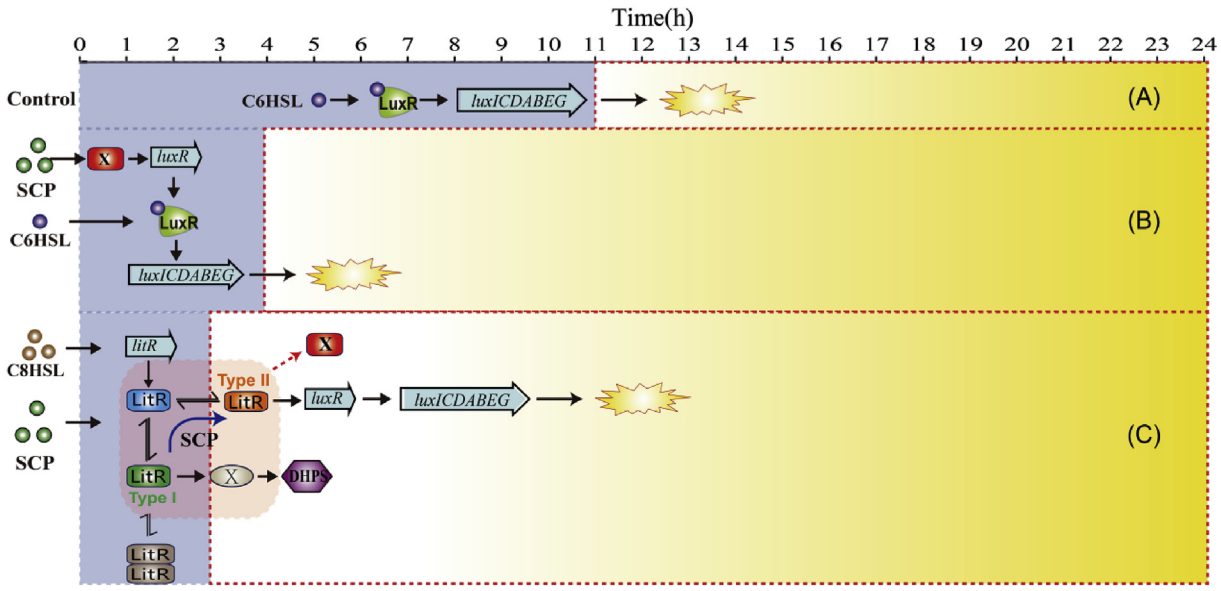

Fig. 6. Mechanisms for the hormesis of SCP, C6HSL and C8HSL on the luminescence. (A) In control groups, the bacteria started emitting light at 11 h; (B) The exogenous C6HSL resulted in an earlier time for the occurrence of the light emitting, and the SCP induced hormesis on luminescence by stimulating the luxR expression; (C) The exogenous C8HSL stimulated the *litR* expression, resulting in an enhancement on the *luxR* expression and thereby the luminescence; SCP changed the LitR forms from type I to type II, resulting in the accumulation of type II and thereby the hormesis on luminescence.

Interestingly, the bacterial growth and the *dhps* expression were also stimulated by the exogenous C8HSL but in an inversed U-shaped mode (Fig. 4I). So it was speculated that LitR would lose (or decrease) its activity in regulating the bacterial growth when its amount exceeded a certain threshold (overexpressed). In previous research (Ramos et al., 2005; Kuttler and Hense, 2008), it was presumed that the LitR proteins can form a homodimer, which is the active form of LitR. Whereas, the current research indicated that the increase in the LitR amount may lead to the inactivation of LitR, probably because LitR at high concentrations formed multimers that are incapable of regulating the bacterial growth (Fig. 6C).

Compared to the single exogenous C8HSL, the mixtures of C8HSL and SCP induced greater stimulation on *luxR* (Fig. 5D), but no greater stimulation on *litR* (Fig. 5E). What's more, the SCP exerted a concentration-dependent stimulation on *dhps* when it was mixed with the exogenous C8HSL (Fig. 5F), although the *litR* amount remained unchanged under this condition (Fig. 5E). So in the mixtures of SCP and exogenous C8HSL, SCP probably activated the inactive forms of LitR multimers.

Taken all of the above results together, we assumed that LitR in *A. fischeri* has two active forms, which regulate the bacterial growth and the luminescence, respectively (see the red shadow in Fig. 6C). The two LitR types were termed as typeI and typeII, respectively. In particular, typeI may lose (or decrease) its activity in regulating the bacterial growth when its content exceeds a certain threshold. What SCP acted on the bacteria was to change the LitR forms from typeI to typeII, resulting in the accumulation of typeII and subsequently leading to hormetic effects on *luxR* as well as the luminescence. This can well explain why SCP could stimulate *luxR* expression (Fig. 5D) without changing the total amount of LitR (Fig. 5E).

The present study reveals a distinct mode of action for SCP towards *A. fischeri*, in addition to its well-known action on DHPS. This mode of action is likely common to SAs, since the hormetic effects on *A. fischeri* luminescence were observed with all the test SAs drugs in this study (Fig. 3). Further work is needed to explore whether this mode of action is universal to other chemicals. This is important because *A. fischeri* luminescence inhibition assay is widely used for toxicity test of chemicals, and this mode of action will not only influence the toxicity results, but also provides us a novel insight into the overall effects of the chemicals on *A. fischeri*.

5. Conclusion

The bacterial language C6HSL and C8HSL were introduced to establish the communication between *A. fischeri* and us. By analyzing the responses of the bacteria under different exposure conditions, a probable mechanism was proposed for the SCP-induced hormesis. It was suggested that LitR in *A. fischeri* have two active forms, type I and type II, which regulate the bacterial growth and luminescence, respectively. The SCP-induced hormesis was due to the interaction between SCP and LitR proteins, which forced the LitR proteins to transform from type I to type II, resulting in the accumulation of type II and subsequently stimulating the luminescence. This work provides a novel insight into the mechanisms for the SCP-induced hormesis a, and gives a valuable reference for the design of novel antibacterial drugs as well as the risk assessment on them.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.10.045.

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